CCL2 and CCL5 are novel therapeutic targets for estrogen-dependent breast cancer

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Abstract

Purpose: Novel therapeutic targets of estrogen receptor (ER)-positive breast cancers are urgently needed because current anti-estrogen therapy causes severe adverse effects, nearly 50% of patients are intrinsically resistant, and the majority of recurrences have maintained ER expression. We investigated the role of estrogen dependent chemokine expression and subsequent cancer growth in human tissues and experimental breast cancer models.

Experimental Design: For in vivo sampling of human chemokines microdialysis was used in breast cancers of women or normal human breast tissue before and after tamoxifen therapy. Estrogen exposure and targeted therapies were assessed in immune competent PyMT murine breast cancer, orthotopic human breast cancers in nude mice, cell culture of cancer cells, and freshly isolated human macrophages. Cancer cell dissemination was investigated using zebrafish.

Results: ER+ cancers in women produced high levels of extracellular CCL2 and CCL5 in vivo, which was associated with infiltration of tumor-associated macrophages. In experimental breast cancer, estradiol enhanced macrophage influx and angiogenesis through increased release of CCL2, CCL5, and vascular endothelial growth factor. These effects were inhibited by anti-CCL2 or anti-CCL5 therapy, which resulted in potent inhibition of cancer growth. In addition, estradiol induced a pro-tumorigenic activation of the macrophages. In a zebrafish model, macrophages increased cancer cell dissemination via CCL2 and CCL5 in the presence of estradiol, which was inhibited with anti-CCL2 and anti-CCL5 treatment.

Conclusions: Our findings shed new light on the mechanisms underlying the progression of ER+ breast cancer and indicate the potential of novel therapies targeting CCL2 and CCL5 pathways.
Translational Relevance

Refined anti-estrogen targeted therapeutics are needed for treatment as well as preventive measures for breast cancer. Our human data, corroborated by experimental models, suggests that CCL2 and CCL5 may serve as novel estrogen dependent targets for both prevention and therapy of breast cancer.
Introduction

In the tumor microenvironment, the interaction between epithelial cells and the stroma is critical for cancer progression (1, 2). Immune cells are a major component of the stroma, and inflammation is considered one of the hallmarks of cancer initiation and progression (3, 4). Immune cells such as tumor-associated macrophages (TAMs) contribute to an environment abundant in chemoattractants and growth factors and are therefore implicated in enhancing cancer growth (5). A high macrophage index in breast cancer has been correlated with tumor progression and poor prognosis (6). However, macrophages within the tissue may be activated into different functional phenotypes that have either anti-tumoral or pro-tumoral properties, and these phenotypes may co-exist in the tumor tissue (3, 5, 7). TAMs are recruited into tissues from monocytes in the circulation by chemoattractants such as CCL2 and CCL5 (8, 9). Various cells in the microenvironment produce these cytokines, and the total abundance of these proteins will define the ability of the tissue to attract inflammatory cells.

Sex steroids, including estrogens, have been found to influence macrophage biology and function through either immunosuppressive or immunostimulatory activity depending on the circumstances (10, 11). In addition, estrogens play an important role in the development and progression of breast cancer and approximately two-thirds of all breast cancers are estrogen receptor (ER)-positive. Blocking the action of estrogens with anti-estrogen treatments such as tamoxifen for 5 to 10 years is a cornerstone in current breast cancer therapy (12). However, this treatment is associated with side effects and the therapy only reduces the risk of recurrence by 30-50% (13). In addition, the mechanisms that underlie hormone-dependent breast carcinogenesis are still unresolved. Hence, identification of estrogen-regulated signaling pathways that could potentially serve as new therapeutic targets for treatment and prevention is urgently needed.
Given the key role of CCL2 and CCL5 in the chemotaxis of macrophages we investigated whether estradiol affected secreted levels of these chemokines in breast tissue. We show that the extracellular in vivo levels of CCL2 and CCL5 in breast cancers of women were 3–5 times higher in the cancerous tissue than in adjacent normal breast tissue, suggesting clinical significance of these cytokines. The tumor tissue also contained an increased macrophage content. In healthy normal breast tissue, the anti-estrogen tamoxifen decreased the in vivo levels of these chemokines. In murine immunocompetent and immunodeficient breast cancer models, 17β-estradiol (E2) stimulated macrophage infiltration via CCL2/CCL5 and induced activation of these macrophages. Tamoxifen abrogated the macrophage influx by reducing CCL2/CCL5 levels and reversed macrophage activation. These results were confirmed by in vitro co-culture of macrophages and breast cancer cells. Treatment with anti-CCL2 and anti-CCL5 antibodies reduced estrogen-dependent breast cancer growth in both murine models. In a zebrafish model, E2 increased formation of metastases when breast cancer cells were co-injected with human macrophages, and this effect was inhibited by anti-CCL2 and anti-CCL5 treatment. Our data reveal previously unrecognized estrogen-dependent pathways in breast tissue, and implicate CCL2 and CCL5 as viable targets in breast cancer therapy.
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Materials and Methods

Microdialysis subjects and experimental design

The regional ethical review board of Linköping approved the study, and all women gave informed consent. Ten postmenopausal women (52–86 years of age), diagnosed with breast cancer, underwent microdialysis before surgery. Before insertion, 0.5 ml lidocaine (10 mg/ml) was administrated intracutaneously. One microdialysis catheter (CMA 71, 10-mm; CMA Microdialysis AB, Solna, Sweden) was inserted intratumorally into the breast cancer and another catheter into adjacent normal breast tissue and connected to a pump (CMA 107; CMA Microdialysis AB) and perfused with 154 mmol/liter NaCl and 60 g/liter hydroxyethyl starch (Voluven; Fresenius Kabi AB, Uppsala, Sweden). Another eighteen postmenopausal women (58–78 years of age) previously treated with surgery for early breast were investigated with microdialysis in their normal unaffected breast. All women had a normal breast examination and normal mammography. Microdialysis was performed before the start of tamoxifen treatment (20 mg/day) and after 6 weeks of treatment. One microdialysis catheter was placed in the upper lateral quadrant of the breast and directed towards the nipple, as previously described, and one was placed in abdominal subcutaneous (s.c.) fat (CMA 71, 20-mm; CMA Microdialysis AB) (14-18). None of the women had taken sex steroid-containing medication for at least 3 months prior to the study. Tamoxifen is not registered as a prevention therapy in Sweden, therefore it was not possible to include a postmenopausal group without previous breast cancer. After a 60-minute equilibration period, the perfusate was collected and stored at -70°C.

Breast tissue culture

Tissue biopsies from reduction mammoplasties, containing epithelium, stroma, and adipose tissue, were produced using an 8-mm biopsy punch (Kai Europe GmbH, Solingen, Germany) and placed in a 12-well cell culture plate (Costar, Cambridge, MA) as previously described.
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(19). Serum-free medium consisting of DMEM/F12 (1:1) with 10 µg/ml transferrin (Sigma-Aldrich), 1 µg/ml insulin (Sigma-Aldrich), and 0.2 mg/ml bovine serum albumin (BSA)(Merck), with or without tamoxifen (10^{-6} M; Sigma-Aldrich) was added. The biopsies were incubated for 7 days at 37°C with 5% CO₂, and the cell culture medium was changed daily. At 7 days, the culture medium was collected and stored at -70°C.

**Cells and culture conditions**

MCF-7 and MDA-MD-231 cells (American Tissue Culture Collection, ATCC; Manassas, USA), authenticated by ATCC, were upon receipt immediately expanded and stored as stocks in liquid nitrogen. A new aliquot was resuscitated for each experiment and never used for more than six months. Cells were maintained in phenol red free DMEM with 2 mM glutamine, 50 IU/ml penicillin-G, 50 µg/ml streptomycin, 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, USA). According to the Swedish ethical legislative guidelines and after obtaining informed consent, venous blood was collected from female donors for isolation of monocytes using Dynabeads®Untouched™ Human monocytes kit (Invitrogen). Monocytes were washed twice and stained with Türk’s solution (Merck, Darmstadt, Germany) and seeded in 12-well cell culture plates, 25,000 cells/cm², incubated for 2.5 hours to allow adhesion. MCF-7 cells, 10,000 cells/cm², seeded on the monolayer of freshly isolated human macrophages and incubated for 24 hours. Cells were treated with 10^{-9} M 17β-estradiol (E2)(Sigma), 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) (Tocris Bristol, UK), 2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN) (Tocris) or 10^{-6} M tamoxifen (Sigma). Hormones were added in serum-free DMEM/F12 (1:1) without phenol red as described above. The medium was changed daily. Culture medium was collected and stored at -70°C. Cells were lysed by repeated freeze-thaw cycles. Total protein concentrations were determined using a Bio-Rad Protein Assay (Bio-Rad, Hercules, USA).

*Mammospheres*
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MCF-7 cells, 2x10^3 cells/well, were cultured in ultra-low attachment plates (Fisher Scientific) in mammocult medium (Stemcell) supplemented with 0.48µg/mL hydrocortisone, 4µg/mL heparin and penicillin-streptomycin. At 500 µm of diameter mammospheres were transferred to 96-well plates coated with 1.5% agarose. Monocytes were isolated as described above and added to mammospheres at 1 x 10^5 monocytes/mammospheres/well and exposed to E2 for 7 days. Mammospheres were fixed with 4% paraformaldehyde and stained using anti-human CD45 primary antibody (Biolegend) 1:200, and Alexa fluor 488 (Abcam) 1:500. A Carl Zeiss Axio Imager with LSM 700 upright microscope was used and numbers of infiltrated monocytes were counted using ImageJ 1.48v.

**Cell migration, proliferation, and detection of angiogenesis factors in vitro**

Monocytes in serum-free medium were placed in a cytoselect migration plate (Cell Biolabs Inc., San Diego, USA), and conditioned medium (CM) from treated MCF-7 cells was used as a chemoattractant with or without human neutralizing anti-CCL2 or anti-CCL5 (5 µg/ml), or goat IgG (R&D Systems). A standard curve of known numbers of monocytes was produced. After 24 hours, fluorescence was measured, Wallac 1420 Victor3 V multi-label plate (PerkinElmer Inc., Massachusetts, USA) with a 485/535 nm filter. Human monocytes were seeded in DMEM containing 5% charcoal-stripped FBS and penicillin and streptomycin. Polarization to M1 and M2 phenotypes was induced by addition of 100 ng/mL IFNγ (Life Technologies, Inc., Gaithersburg, MD) or 20 ng/mL IL-4 (Life technologies, Inc.) for 24 hours.

**Murine breast cancer models**

Animal care and treatment conformed to regulatory standards. The institutional animal ethics committee at Linköping University approved the study. Female athymic nude mice, Balb/C-nu/nu, and FVB/N mice (6–8 weeks old, Scanbur, Sweden) were housed in individually ventilated cages (IVCs) with a light/dark cycle of 12/12 hours, with rodent chow and water
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ad libitum. Mice were anesthetized by intraperitoneal (i.p.) injection of ketamine/xylazine and oophorectomized prior to subcutaneous (s.c.) implantation with 3-mm pellets containing 17β-estradiol (0.18 mg/60-day release; Innovative Research of America, Sarasota, USA) resulting in serum concentrations of 150 to 250 pM (20, 21). One week after surgery, MCF-7 cells (5 × 10^6 cells in 200 µl phosphate buffered saline (PBS)) were injected into the dorsal mammary fat pad using a similar approach to the original pre-clinical studies using tamoxifen (22). Because the MCF-7 explants require estrogen for tumor formation and growth, a non-estrogen control group is impossible to achieve using this in vivo model. The FVB/N mice were injected in the dorsal mammary fat pad with tumor cells (1 × 10^6 cells in 200 µl PBS) derived from a transgenic mouse strain expressing polyoma middle T (PyMT) antigen under the control of the mouse mammary tumor virus (MMTV) long terminal repeat (23). These mice develop spontaneous adenocarcinomas of all mammary epithelium by 8 to 10 weeks of age. Tumors were excised, dissociated in a collagenase/dispase solution (100 ml PBS with 25 mg collagenase/250 mg dispase; Roche, Nutley, USA) to generate a single-cell suspension and cultured until confluence. We, and others, have shown that these breast cancers express the estrogen receptor (ER) at significant levels at early carcinoma stages and that ER expression decreases at later stages (24, 25). Harvesting tumor tissue at early stages results in estrogen-dependent cancer growth in the syngeneic recipient mouse as previously described (21, 25). When the tumor size reached 20 mm^2, the mice were divided into different treatment subgroups: tamoxifen (1 mg/mouse/every second day, s.c), anti-CCL2, anti-CCL5, or isotype control (20 µg intratumorally, twice a week, R&D Systems Minneapolis, MN, USA).

Microdialysis of mice

Mice were anesthetized with an i.p. injection of ketamine/xylazine. Body temperatures were maintained using a heat lamp. Microdialysis catheters (CMA 20, 100-kDa cutoff; CMA Microdialysis AB) were inserted into tumor tissue and connected to a pump (CMA 102;
CMA Microdialysis AB) perfused at 0.6 μl/min with Voluven 60% (Fresenius Kabi AB) as previously described (26, 27). After a 60-minute equilibrium period, outgoing microdialysates were collected and stored at -70°C for subsequent analysis.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded tumors cut in 4 μm sections, de-paraffinized, and exposed to rat anti-mouse F4/80 (Abcam, Cambridge, USA), rat on mouse HRP Polymer Kit (BioCare Medical, Concord, USA), rabbit anti-human von Willebrand factor, (Dako, Carpinteria, CA), or mouse anti-human CD68 (Dako) antibodies and counterstained with Mayer’s hematoxylin. Negative controls did not show staining. Images of three areas of each tumor section from 4–8 individuals in each treatment group were acquired on an Olympus BX43F microscope (×400 magnification; Lund, Sweden) and digitally analyzed and quantified using Olympus CellSens Dimension software.

For immunofluorescence, sections were exposed to rat anti-mouse F4/80 (Cl:A3-1; Abcam), rabbit anti-human iNOS (Abcam), or mouse anti-MRC1 (Abcam) antibodies, and incubated with conjugated antibodies (Alexa fluor 546, 594, and 488) mounted using SlowFade Gold containing 4’,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Samples were visualized using an Olympus BX41 light/fluorescence microscope (×40/0.75 magnification) with excitation filters of 495–519 nm and 556–573 nm, using an Olympus DP70 CCD camera. The images were digitally analyzed with Olympus CellSens Dimension software.

**Zebrafish experiments**

The institutional animal ethics committees at Linköping University and Karolinska Institute approved all zebrafish experiments. MCF-7 cells were cultured with or without 10⁻⁹ M E2, and labeled with 1,1’-dioctadecyl-3,3,3’3’-tetramethylindocarbocyanine (DIL) as previously described (28). Freshly isolated human monocytes were diluted in DMEM with 1% FBS with or without 10⁻⁹ M E2 and mixed with MCF-7 cells at a final concentration of 10%
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monocytes. For cell injections containing antibodies, goat anti-human CCL2, goat anti-human CCL5 or isotype control, normal goat IgG (R&D Systems Minneapolis, MN, USA), were added at a final concentration of 5 µg/mL. Transgenic Tg(fli1:EGFP)y1 zebrafish embryos (ZIRC, Eugene, OR, USA) were raised in E3 medium supplemented with 1-phenyl-2-thiourea (PTU) and cells were implanted into the perivitelline space of 2 day-old zebrafish embryos, as previously described (28). The injected embryos were placed in E3 embryo medium containing 0.2 × 10⁻³ M PTU and 1 × 10⁻⁹ M E2. After 3 days, the embryos were euthanized by an overdose of 0.04% MS-222 (Sigma-Aldrich), and mounted in VectaShield (Vector, Burlingame, CA USA). Cancer cells were visualized under a fluorescence microscope (Nikon D-eclipse C1, Tokyo Japan). Cells present posterior to the anal opening were counted.

Quantification of proteins

Microdialysates and cell culture media were analyzed for proteins using quantitative immunoassay ELISA kits or fluorokine MAP base kits (R&D Systems) analyzed on a Luminex 100 System (Luminex, Austin, USA) according to the manufacturer’s guidelines.

Statistical analyses

Data are expressed as means ± SEM. Wilcoxon signed rank test for paired observations, Student’s t-test, or Spearman’s correlation were used as appropriate with Prism 6.0 (GraphPad Software, San Diego, USA).
Results

Extracellular CCL2 and CCL5 in human breast tissue in vivo

Microdialysis in breast cancers of women before surgery was performed without complications as previously described (29-32). Characteristics of the subjects are presented in Supplementary Table 1. The extracellular in vivo levels of CCL2 and CCL5 were 3-5 times higher within the breast cancer than in adjacent normal breast tissue (Fig. 1A), and the levels correlated significantly with plasma concentrations of estradiol, r=0.659, p=0.04 for CCL2 and r=0.705, p=0.03 for CCL5. Macrophage staining revealed significantly increased macrophage infiltration in the cancerous tissue compared with normal breast tissue as shown in (Fig. 1A).

Microdialysis performed in normal human breast tissue and subcutaneous fat of women showed that the levels of CCL2 and CCL5 in breast tissue significantly decreased after 6 weeks of tamoxifen therapy whereas the levels in fat tissue were unchanged (Fig 1B). This was verified ex vivo where normal human breast tissue biopsies exposed to tamoxifen exhibited significantly decreased levels of CCL2 and CCL5 after tamoxifen exposure (Fig 1C).

E2 increased extracellular CCL2 and CCL5 in vivo and macrophage infiltration into experimental breast cancer

We set up two different experimental breast cancer models, the immune competent polyoma middle T (PyMT) antigen model and MCF7 human breast cancer in nude mice to further explore possible E2 effects on macrophage infiltration. As shown in Fig. 2, E2 induced cancer growth in PyMT tumors and tamoxifen abrogated growth of the MCF-7 tumors (Fig. 2A-B). Microdialysis of these experimental breast cancers revealed that E2 significantly increased intratumoral levels of CCL2 and CCL5 in vivo in PyMT tumors (Fig. 2A). In the MCF-7 tumors consisting of human cancer cells in a murine stroma, tamoxifen significantly
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decreased cancer cell-derived (human) CCL5 levels and stroma-derived (murine) CCL2 levels (Fig. 2B). Human CCL2 and murine CCL5 were below detection levels. However, E2 induced a massive influx of macrophages into PyMT tumors compared to controls (Fig. 2A), whereas tamoxifen-treated MCF-7 tumors exhibited significantly lower numbers of infiltrating macrophages than tumors exposed to E2 only (Fig. 2B). These results were confirmed in ER+ ZR75-1 breast cancer tumors and ER+ ovarian BG-1 tumors in nude mice (supplementary Fig. S2). An infrequent neutrophil infiltration was observed in both tumor models without any differences between treatment groups (supplementary Fig. S1).

To elucidate the role of different ERs in the regulation of CCL2 and CCL5 expression we exposed MCF-7 cells, which express both ERα and ERβ, and MDA-MB-231 cells that express ERβ but not ERα, to selective agonists. In MCF-7 cells the selective ERα agonist PPT increased CCL2 and CCL5 expression in a similar fashion as E2 whereas the levels were unaffected after treatment with DPN, a selective ERβ agonist. In MDA-MD-231 cells treated with E2 or DPN did not change the chemokine levels compared with control cells (Fig. 2C). In tumors the cancer cells grow in close interaction with the stroma, which in the case of breast tumors consists predominantly of macrophages. Co-cultures of MCF-7 cells and human macrophages and obtained similar results to those obtained in vivo: E2 significantly increased the levels of CCL2 and CCL5, which were counteracted by tamoxifen (Fig. 2D). The E2 dependent infiltration was further confirmed in mammospheres incubated with human monocytes, (Fig. 2E). Conditioned medium (CM) from E2-treated MCF-7 breast cancer cells also significantly increased the migration of macrophages whereas tamoxifen totally abrogated this effect (Fig. 2F). Addition of neutralizing antibodies against CCL2 and CCL5 to the CM repressed the migration, confirming the key role of CCL2 and CCL5 in E2-induced migration of macrophages (Fig. 2F). Other chemoattractants such as granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-
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CSF), and epithelial-derived neutrophil-activating peptide 78 (ENA-78) were not detectable in our hands in the MCF-7 cells. CCL2 exerts its action via CCR2 and CCR4 receptors whereas CCR1 and CCR5 are the main receptors for CCL5 (33). In our experimental model we detected expression of CCR2 and CCR4, whereas CCR1 was undetectable and CCR5 exhibited weak staining in the PyMT tumors and almost no staining in the MCF tumors (Supplementary Fig. S3).

**Macrophage activation in breast cancer explants**

Because macrophages can be activated into different phenotypes, simplistically termed M1 and M2, we next investigated whether various treatments affected their phenotype. Staining with the M2 marker MRC1 and the M1 marker iNOS revealed that the majority of macrophages in PyMT and MCF-7 tumors exposed to E2 were MRC1-positive and iNOS-negative, whereas tamoxifen potently decreased the expression of MRC1 and upregulated iNOS (Fig. 3A-B). Microdialysis of the cancers revealed an *in vivo* cytokine profile suggestive of induction of a pro-tumorigenic environment by E2 in the PyMT tumors, with a significant decrease in interleukin-1 receptor (IL-1Ra) and a borderline significant decrease in IL-6 (Fig. 3A). In the MCF-7 tumors, tamoxifen induced anti-tumorigenic activation of the stroma with increased levels of murine IL-1Ra and IL-6 (Fig. 3B). Tumor necrosis factor (TNF-α), IL-10, and IL-2 were not detectable. Pro-tumorigenic macrophages also secrete high levels of vascular endothelial growth factor (VEGF) (34) and we found increased levels of VEGF *in vivo* in E2-treated PyMT tumors whereas tamoxifen decreased the levels of cancer cell- and stroma-derived VEGF in the MCF-7 tumors (Fig. 3C). To further investigate this finding *in vitro*, MCF-7 cells were cultured with medium from freshly isolated human macrophages that were exposed to IL-4 (M2 polarized) or interferon-gamma (IFNγ) (M1 polarized). Cancer cells grown in presence of M2 polarized medium exhibited significantly...
increased VEGF secretion compared with those grown in medium from M1 macrophages, and this effect was further enhanced in the presence of E2 (Fig. 3C).

Medium from M1 macrophages decreased proliferation of MCF-7 cells, whereas medium from M2 macrophages increased proliferation, compared with control medium (control medium [0.28±0.008] vs. M1 medium [0.23±0.003], p<0.01, and control vs. M2 medium [0.39±0.01], p<0.001).

**Hormone-dependent macrophage activation in vitro**

To further verify our *in vivo* findings, freshly isolated human macrophages that express the estrogen receptor (27, 35, 36) from female donors were cultured *in vitro* alone or in coculture with MCF-7 breast cancer cells with direct cell surface contact. When macrophages were cultured alone, E2 exposure led to a significant decrease in IL-1Ra and a significant increase in the levels of IL-6 and TNF-α (Fig. 4). Addition of tamoxifen reversed these changes and significantly increased the levels of IL-1β (Fig. 4). When macrophages were cocultured with MCF-7 cells, the profile of the secreted cytokines was changed and exhibited a similar pattern to that observed in the *in vivo* microdialysis, with a significant increase in the secretion of IL-1β, IL-1Ra, and IL-6 induced by tamoxifen. Moreover, IL-1α and TNF-α were induced by tamoxifen (Fig 4). IL-10, IL-12, CCL17, and CCL22 were undetectable in all samples.

**E2 induced dissemination of MCF-7 cells via CCL2 and CCL5 in the presence of macrophages in zebrafish**

Metastasis is the major cause of death in breast cancer patients. However, there are no experimental breast cancer models in which ER+ breast cancer spontaneously metastasizes with maintained ER expression (24, 37). Therefore, we used the zebrafish metastases model to further explore whether the increase in CCL2 and CCL5 induced by E2 affected the dissemination of cancer cells. Without the addition of freshly isolated human monocytes, E2
stimulation of MCF-7 cells did not increase dissemination to distal regions in the zebrafish. However, dissemination of the cancer cells increased significantly in the presence of monocytes. Interestingly, addition of E2 to the mixture of MCF-7 cells and macrophages further increased macrophage migration (Fig 5A) and this E2-dependent increase in migration was counteracted by treatment with anti-CCL2 and anti-CCL5 antibodies (Fig. 5B). Both antibody treatments were equally effective and combination therapy did not increase the effect.

**Anti-CCL2 and anti-CCL5 inhibited E2-stimulated tumor growth**

To test whether inhibition of CCL2 and CCL5 alone or in combination affected estrogen-stimulated cancer growth we treated mice bearing MCF-7 or PyMT breast cancers with the corresponding antibodies. In both tumor models, blockade of these chemokines significantly reduced estrogen-stimulated cancer growth. In the PyMT model a combination of antibodies against CCL2 and CCL5 significantly decreased tumor growth compared with either treatment alone (Fig. 6A) whereas in MCF-7 cancers the combination of anti-CCL2 and anti-CCL5 antibodies was not better than either treatment alone (Fig. 6B). Antibody treatment resulted in significantly decreased infiltration of macrophages in the PyMT model (Fig. 6C) and significant decrease in microvessel area (Fig. 6D). The treated tumors from MCF-7 breast cancers were too small to perform meaningful immunohistochemical analysis.
Discussion

Here, we report a novel mechanism by which estrogen affects the communication between cancer cells, macrophages, and tumor blood vessels leading to increased cancer growth and metastasis (Supplementary Fig. S4). We found that the extracellular in vivo levels of CCL2 and CCL5 were 3–5 times higher in the breast cancer tissue of women compared with normal adjacent breast tissue and correlated with E2 levels. These cancers also exhibited increased infiltration of macrophages. Tamoxifen downregulated the extracellular CCL2 and CCL5 levels in normal human breast tissue in vivo. In experimental breast cancer models, E2 increased CCL2 and CCL5 levels with concomitant massive influx of macrophages, which was counteracted by tamoxifen. In addition to stimulation of migration of macrophages, E2 induced activation of these cells into a tumor-promoting phenotype both in tumor explants and in vitro. In mice, blocking CCL2 and CCL5 by antibody treatment significantly decreased estrogen-stimulated breast cancer growth and angiogenesis. In the zebrafish, macrophages increased the ability of cancer cells to disseminate to the periphery, which was significantly enhanced in presence of E2. Again, blocking CCL2 and CCL5 abolished E2-stimulated cancer cell dissemination.

A critical factor for the development and progression of breast cancer is exposure to estrogen (38). Anti-estrogen therapy reduces the risk of primary breast cancer and recurrence but this long-term treatment is associated with severe side effects such as thromboembolic events, endometrial cancer, joint pain, and low quality of life (13, 39). This emphasizes the need to identify novel estrogen-dependent targets for improved therapy and cancer prevention. The close interaction between cancer cells and the surrounding stroma is key in carcinogenesis and it has recently been reported that the outcome of cancer therapy is affected by the tumor-stroma interaction (40, 41). Macrophages constitute a major component of the stroma in breast cancer (1, 2), but the role of estrogens in macrophage migration and
function in breast cancer has not previously been fully explored. Our present data strongly suggest that one of the growth promoting effects of E2 in hormone-dependent breast cancer is a massive influx of macrophages, as demonstrated in both an immune competent murine breast cancer model and in nude mice bearing ER-positive human breast tumors. Tamoxifen has previously been shown to reduce the number of macrophages in rat mammary glands (40); however, this is the first time that the inhibitory effect of tamoxifen on macrophage infiltration has been demonstrated in breast cancer. In addition, we show that E2 induced macrophage influx into experimental ovarian cancer, suggesting similar mechanisms in hormone-dependent cancers of different origins.

CCL2 and CCL5 play critical roles in the recruitment and activation of monocytes/macrophages and might be associated with advanced disease and poor prognosis of breast cancer (42, 43). These chemokines can be expressed by many cell types in tissues and the total amount of these proteins in the extracellular space, the bioactive site for these chemokines, will define the physiologic condition of the microenvironment (9). To the best of our knowledge, this is the first report of measurement of these chemokines in the extracellular space in human tissues. In addition to the upregulation of both CCL2 and CCL5 in breast cancers of women, the levels of these chemokines correlated significantly with the level of E2. These results were corroborated in experimental breast cancer models, in which E2 significantly increased the levels of CCL2 and CCL5 in vivo. This effect may be a result of a direct gene regulation via an estrogen responsive element as computational data on large genome data sets suggest an ERα binding site in the promoter region of the CCL2 (44). In addition, E2 may indirectly affect chemokine expression by interacting with the transcription factors AP-1 and NF-κB that bind to promoter regions of CCL2 and CCL5 (45-47). Tamoxifen down-regulated CCL2 and CCL5 in normal breast tissue in postmenopausal women in vivo, a result confirmed by ex vivo culture of human breast tissue biopsies,
Chemokines in human breast cancer suggesting that these proteins play a physiologic role in normal breast tissue. Whether this down-regulation also occurs after therapy with aromatase inhibitors or in premenopausal women using tamoxifen remains to be elucidated. Antibody therapy against these proteins significantly inhibited estrogen-driven breast cancer growth in both murine models, supporting a key role of these chemokines in estrogen-dependent cancer growth.

One major difficulty in studying estrogen-dependent metastasis formation is the lack of experimental models in which the ER is maintained during dissemination of the cancer cells from a primary tumor. In the PyMT model ER is expressed during early stages of cancer development in the mammary gland but is lost at the metastasis stage (24). ER-expressing human breast cancer cell lines such as MCF-7 and ZR-75-1 can form primary cancers in nude mice but these tumors do not metastasize. As recently reported, approximately two-thirds of ER-expressing primary breast cancers maintain the ER at the metastatic site and nearly 30% of initially ER-negative primary breast cancers gain ER expression in the metastatic lesion (48). These human data highlight the importance of exploring estrogen-dependent breast cancer dissemination. Because of the lack of ER+ metastasis models we chose to study the role of macrophages in the cancer cell dissemination process in the zebrafish system, which we have developed as a metastasis model (28). The advantage of this approach is that it allows detailed insights into the dissemination process at a single cell level. Using this system we showed that, in addition to the effects of estrogen on primary breast cancer growth, E2 increased the ability of cancer cells to disseminate into the periphery in the presence of macrophages. The important role of estrogen-induced CCL2 and CCL5 was confirmed by blocking these chemokines, which abrogated the E2-dependent cancer cell dissemination. These results suggest that estrogen-dependent release of CCL2 and CCL5 might be important in later stages of breast cancer progression; i.e., during the establishment of metastatic disease, which is the most detrimental stage for patient outcome.
Infiltration of tumor-associated macrophages per se is strongly associated with tumor progression and metastasis (5, 7). Nevertheless, recent data show that TAMs may be either anti-tumorigenic or pro-tumorigenic depending on their phenotype, and both types of macrophage co-exist in cancer tissues (7, 49). In brief, anti-tumorigenic (M1) macrophages secrete elevated levels of nitric oxide via inducible nitric oxide synthase (iNOS), together with IL-1, IL-6, IL-12, and TNF-α, whereas pro-tumorigenic macrophages (M2) display high levels of mannose receptor-1 (MRC1), IL-10, CCL2, CCL17, CCL22, and angiogenic factors including VEGF (7, 49, 50). However, macrophage skewing displays great heterogeneity and plasticity, and populations are rarely pure M1 or M2. Tumors from our murine models that were exposed to E2 exhibited mainly MRC1+ macrophages and low levels iNOS, whereas tumors that were grown in an estrogen-depleted environment or exposed to tamoxifen exhibited MRC1-negative and iNOS-positive macrophages. These immunohistochemical stainings and our in vivo cytokine profile data strongly suggest that hormones have the ability to change the phenotype of intratumoral macrophages. Tamoxifen induced an M1-like skewing of the cytokines, including IL-1β expression. Although IL-1β is among the M1 markers this cytokine has been shown to exhibit pro-angiogenic activity in various cancers, including breast cancer (51, 52). However, the tamoxifen-induced increase in IL-1β was associated with a concomitant increase in the level of IL-1Ra, a natural inhibitor of IL-1, to a higher extent than that of IL-1β, leading to inactivation of IL-1β. This is consistent with the anti-tumorigenic property of this cytokine that we, and others, have recently described (27, 53).

Macrophages, especially the M2 type, are also a source of other angiogenic factors in the microenvironment, such as VEGF (50). In addition to tumor regression, treatment with antibodies against CCL2 and CCL5 resulted in significantly decreased microvessel density, thus corroborating the link between macrophage infiltration and
Chemokines in human breast cancer angiogenesis. *In vitro*, we further confirmed the critical role of M2 macrophages in the release of VEGF by showing that their medium increased secretion of VEGF from cancer cells compared with medium from M1 macrophages, an effect that was enhanced by E2. This suggests that increased release of angiogenic factors by M2 macrophages is one of the mechanisms involved in E2-dependent breast cancer growth.

In summary, our data suggest that hormonal regulation of CCL2 and CCL5 is a key factor in estrogen-dependent breast cancer progression. Macrophages that are attracted by these chemokines play an important role in cancer progression and depletion of these TAMs or re-direction of detrimental TAMs to a beneficial phenotype may represent a sophisticated treatment strategy for breast cancer. To our knowledge, our data reveal previously unrecognized effects of estrogens and tamoxifen in breast cancer and identify CCL2 and CCL5 as two estrogen-induced targets that may be exploited therapeutically. The profound remodeling of the microenvironment by estrogens and anti-estrogens described here provides insights into novel therapeutic strategies for hormone-dependent breast cancer.
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References:


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Figure Legends

Figure 1 Extracellular CCL2 and CCL5 levels in human breast tissue in vivo

A. Ten postmenopausal breast cancer patients underwent microdialysis before surgery. One catheter was inserted into the breast cancer and another into adjacent normal breast tissue and CCL2 and CCL5 levels in the microdialysates were analyzed. There were significant correlations between plasma estradiol (E2) and tumor CCL2 and CCL5 and between tumor CCL2 and tumor CCL5 by Spearman’s correlation coefficient. Immunostaining of the macrophage marker CD68 was performed on breast cancer tissue and adjacent normal breast tissue and the stained area was quantified as described in Materials and Methods. Representative sections from each patient are shown, scale bar=20 µm. Graphed data are presented as mean ± SEM.

B. Eighteen women underwent microdialysis of unaffected normal breast tissue; in 17 of these women abdominal subcutaneous fat was also analyzed before and 6 weeks after initiation of tamoxifen (Tam; 20 mg/day) as an adjuvant treatment for early breast cancer. Graphed data are presented as mean ± SEM.

C. Normal human breast tissue biopsies from women undergoing reduction mammoplasty were cultured in vitro with or without tamoxifen for 7 days, and the medium was analyzed for CCL2 and CCL5 content, n=19. Graphed data are presented as mean ± SEM.
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Figure 2. Estradiol increased extracellular CCL2 and CCL5 in vivo and macrophage infiltration in experimental breast cancer models.

A. Oophorectomized FVB/N mice supplemented with physiological levels of E2 (n=9) or placebo (n=7) were injected with PyMT tumor cells in the mammary fat pad and tumor growth was monitored using a caliper, ***P<0.001.

Microdialysis was performed in the PyMT breast cancer explants and levels of murine CCL2 and CCL5 were measured (n=7 in each group). Tumor sections were stained for macrophages (F4/80) and the stained area was quantified as described in Materials and Methods. Representative sections from each treatment group are shown, scale bars=50 μm. Graphed data are presented as mean ± SEM.

B. Oophorectomized Balb/C-nu/nu mice were supplemented with physiological levels of E2 and injected with MCF-7 cells in the mammary fat pad. At similar tumor sizes, one group continued with E2 treatment and the other group received an additional tamoxifen treatment (1 mg/mouse every second day s.c.) (n=10 in each group), ***P<0.001.

Microdialysis was performed in the MCF-7 breast cancer explants and the amounts of cancer cell-derived (human) and stroma cell-derived (murine) CCL2 and CCL5 were measured. Tumor sections were stained for macrophages (F4/80) and the stained area was quantified as described in Materials and Methods. Representative sections from each treatment group are shown; scale bars=50μm. Graphed data are presented as mean ± SEM.

C. Cultured MCF-7 cells (ERα+ and ERβ+) and MDA-MB-231 cells (ERα- and ERβ+) were exposed to E2 10⁻⁹M, tamoxifen 10⁻⁶M (T), PPT 10⁻⁹M, or DPN 10⁻⁹M and cell culture media was analyzed for CCL2 and CCL5. Graphed data are presented as mean ± SEM.

D. Co-culture of MCF-7 breast cancer cells and freshly isolated human macrophages that were exposed to E2 and tamoxifen (T) for 24 hours. Graphic data are presented as mean ± SEM.
E. Mammospheres were cultured and at similar sizes freshly isolated monocytes were added. After 7 days of culture staining and counting of infiltrated macrophages were performed of controls and E2 treated mammospheres. Graphed data are presented as mean ± SEM.

F. *In vitro* migration assay of freshly isolated human macrophages towards conditioned medium from MCF-7 cells exposed to E2, tamoxifen (T), and E2 with or without antibodies against CCL2 and CCL5. Graphed data are presented as mean ± SEM.
Figure 3. Estradiol induced macrophage activation in breast cancer explants.

A. Oophorectomized FVB/N mice supplemented with physiologic levels of estradiol (E2) or placebo were injected with PyMT tumor cells in the mammary fat pad. Tumor sections were stained for macrophages (F4/80) (red) and the M2 macrophage marker, MRC1 (green), or iNOS (green). The percentage of cells positive for MRC1 or iNOS was quantified as described in Materials and Methods. Representative sections from each treatment group are shown, scale bars=20 μm. Microdialysis was performed in the PyMT breast cancer explants and murine IL-1β, IL-1Ra, and IL-6 was measured. Graphed data are presented as mean ± SEM.

B. Oophorectomized Balb/C-nu/nu mice were supplemented with physiological levels of estradiol (E2) and injected with MCF-7 cells in the mammary fat pad. At similar tumor sizes, one group continued with E2 treatment and the other group received an additional tamoxifen (T) treatment (1 mg/mouse every second day s.c.). Tumor sections were stained for macrophages (F4/80) (red) and the M2 macrophage marker, MRC1 (green), or iNOS (green). The percentage of cells positive for MRC1 or iNOS was quantified as described in Materials and Methods. Representative sections from each treatment group are shown, scale bars=20 μm. Microdialysis was performed in the MCF-7 breast cancer explants and stromal cell-derived (murine) IL-1β, IL-1Ra, and IL-6 were measured. Graphed data are presented as mean ± SEM.

C. Intratumoral microdialysis for sampling of murine VEGF in PyMT cancers supplemented with physiologic levels of E2 or placebo (E2 and Control) and cancer cell-derived (human) and stromal-derived (murine) VEGF in MCF-7 breast cancers in nude mice exposed to E2 or E2+Tamoxifen (E2+T) (three left panels).
MCF-7 cells were grown in the presence of culture medium from M1 and M2 differentiated human freshly isolated macrophages with or without $10^{-9}$ M estradiol. Secreted VEGF was measured (right panel). Graphed data are presented as mean ± SEM.
Figure 4. Estradiol affected the secreted cytokine profile of human macrophages.

Freshly isolated human monocytes were cultured alone (left panels) or in co-culture with human MCF-7 breast cancer cells (right panels) and exposed to estradiol (E2) and tamoxifen (Tam) for 24 hours. Cytokines were measured using ELISA, n=4-6 in each group. Graphed data are presented as mean ± SEM.
Figure 5. In the presence of human monocytes, estradiol increased breast cancer cell dissemination to the periphery via CCL2 and CCL5.

A. Zebrafish embryos were injected with MCF-7 cells with or without human monocytes and with or without estradiol (E2), as described in the materials and methods section, and the number of disseminated cells was counted (n=12-31 in each group). Graphed data are presented as mean ± SEM.

B. Zebrafish embryos were injected with MCF-7 cells and human monocytes and treated with estradiol (E2), as described in the materials and methods section. Anti-CCL2, anti-CCL5, or isotype control IgG antibody (ISO) (5 µg/ml) was also added to the mixture injected into the zebrafish embryo. The number of disseminated cells was counted (n=5–18 in each group). Graphed data are presented as mean ± SEM.
Figure 6. Anti-CCL2 and anti-CCL5 antibodies reduced estrogen-dependent breast cancer growth in mice.

A. Oophorectomized FVB/N mice were supplemented with a physiological level of estradiol (E2) and injected with PyMT tumor cells in the mammary fat pad. Anti-CCL2, anti-CCL5, or their combination (anti-CCL2/CCL5) treatment was administered concomitant with E2 supplementation as described in Materials and Methods, ***\(P<0.001\) compared isotype control IgG antibody (ISO) and ##\(P<0.01\) compared to anti-CCL2 or anti-CCL5 treatment, n=7 in each group.

B. Oophorectomized Balb/C-nu/nu mice were supplemented with physiologic levels of E2 and injected with MCF-7 cells in the mammary fat pad. Anti-CCL2, anti-CCL5, or their combination (anti-CCL2/CCL5) treatment was administered concomitant with E2 supplementation as described in Materials and Methods. ***\(P<0.001\) compared to control group, isotype control IgG antibody (ISO), n=6–8 in each group.

C. Macrophage content (F4/80 staining) of PyMT cancers treated with anti-CCL2 and anti-CCL5 antibodies. Scale bars=20 μm. Graphed data are presented as mean ± SEM.

D. Microvessel area (von Willebrand staining) of PyMT cancers treated with anti-CCL2 and anti-CCL5 antibodies. Scale bars=20 μm. Graphed data are presented as mean ± SEM.
Figure 1: Graphs showing the extracellular CCL2 and CCL5 levels in different conditions. The bar charts illustrate the comparison between normal breast and breast cancer tissues, as well as the effects of Tamoxifen treatment. The significance levels (P-values) indicate the statistical difference between conditions.
Figure 2
Figure 4
Figure 5
Figure 6

(A) Tumor area (PyMT) (mm²) over time (days) for different treatments: E2±IgG, E2±Anti-CCL2, E2±Anti-CCL5, E2±Anti-CCL2/CCL5. The graph shows a significant increase in tumor area over time, with a comparison between E2±IgG and E2±Anti-CCL2/CCL5 indicated by ***.

(B) Tumor area (MCF-7) (mm²) over time (days) for different treatments: E2±IgG, E2±Anti-CCL2, E2±Anti-CCL5, E2±Anti-CCL2/CCL5. The graph shows a significant increase in tumor area over time, with a comparison between E2±IgG and E2±Anti-CCL2/CCL5 indicated by ***.

(C) Comparison of Macrophages (% area F4/80+) among different treatments: ISO, Anti-CCL2, Anti-CCL5, Anti-CCL2+ Anti-CCL5. The bar graph indicates a significant difference between ISO and Anti-CCL2/CCL5 treatments, with a p-value < 0.0001. Representative images show staining for F4/80+ expression in PyMT ISO, Anti-CCL2, Anti-CCL5, and Anti-CCL2+ Anti-CCL5 groups.

(D) Comparison of Microvascular area (%) among different treatments: ISO, Anti-CCL2, Anti-CCL5, Anti-CCL2+ Anti-CCL5. The bar graph indicates a significant difference between ISO and Anti-CCL2/CCL5 treatments, with a p-value < 0.0001. Representative images show staining for microvessels in PyMT ISO, Anti-CCL2, Anti-CCL5, and Anti-CCL2+ Anti-CCL5 groups.
CCL2 and CCL5 are novel therapeutic targets for estrogen-dependent breast cancer


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