Estradiol represses IRF3-7 signaling pathways in ER+ astrocytes to suppress immune surveillance during early brain metastatic colonization

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ABSTRACT

While estradiol (E2) -the main premenopausal ovarian hormone-, is best known for its mitogenic role in estrogen-receptor positive (ER+) breast cancer, we have shown that ovarian and brain-synthesized 17-□-estradiol (E2) promote brain metastasis of ER negative (ER-) tumors through effects on the microenvironment. Aromatase, the key enzyme for E2 synthesis, is expressed and active in reactive astrocytes and other cells within the brain, and brain synthesized E2 can act in a neurotransmitter-like manner playing pleotropic effects in brain function. Thus, ovarian and brain estrogens may contribute to brain metastatic progression. **Objective**: To determine the extent to which E2 and aromatase function modulate the neuroinflammatory function of astrocytes and their impact to brain metastatic colonization. **Methods and Results:** Immunohistochemistry analysis of brains from mice carrying primary ER¯ tumors in the mammary fat pad showed that astrocytes become activated (GFAP+) and express ERα and aromatase since early stages of spontaneous metastatic dissemination. Aromatase-expressing astrocytes are abundant in clinical brain metastasis and in experimental models of BCBMs in rodents, suggesting ovarian and brain E2 accompany brain metastasis seeding and outgrowth. Human and murine astrocytes express aromatase and synthesize E2 in vitro, and aromatase inhibitors (ie. letrozole) abolishes this synthesis. To assess how E2 or aromatase inhibition modulate astrocytic function, primary astrocytes were treated with E2, vehicle or Letrozole for 24hr and analyzed using global RNAseq. Upstream regulator analysis of differentially expressed genes showed that E2 represses a program of pro-inflammatory chemokines in the Interferon-Regulatory factor-3 and 7 pathways (IRF-3, P=1.93 x 10-20; IRF-7 P=1.84.93 x 10-18); and activates the transcriptional coactivator TRIM24 (P=1.31 x 10^25). Consistently, E2-treated astrocytes showed decreased p-IRF3 and p-IRF-7 levels compared to letrozole-treated astrocytes in vitro. IRF-3 and 7 are key transcriptional regulators of type-I interferon (IFN), which drive anti-tumoral immune responses through regulation of C-C Chemokines. Consistently, E2-treated astrocytes expressed lower mRNA and protein levels of a program of chemokines compared to letrozole-treated astrocytes. Since these chemokines are required for activation and polarization of microglia, we next determined whether E2-treated astrocytes had differential ability to activate and polarize primary microglia. Direct treatment of microglia with E2 or letrozole did not significantly shift their activation or polarization markers, however, treatment of microglia with conditioned media (CM) from letrozole-treated astrocytes increased the fraction of CD16/32 M1 (tumors suppressive microglia) and decreased the fraction of CD206+ M2 (tumor promoting microglia) compared to treatment with CM-E2 treated astrocytes. These data suggest that E2 suppression of IRF3/7 pathways in astrocytes, leads to suppression of chemokines required for early activation of an anti-tumoral microglia response. Accordingly, analysis of cancer disseminated cells in the brain early after hematogenous dissemination of 231BR cancer cells showed an increased number of single surviving cancer cells in the brain parenchyma in E2-treated mice compared to OVX or OVX+letrozole treated mice. **Conclusion:** These data support the notion that E2 represses immune-surveillance in the early brain metastatic niche in favour of brain metastatic colonization, and that aromatase inhibitors may reactivate innate anti-tumoral responses at early stages of metastatic dissemination.

BACKGROUND

- Pre-menopausal women have higher risk of BM[1,2]. Up to 75% of women with TNBC are pre-menopausal, and these women have increased incidence of brain metastases (58%) compared to post-menopausal women (27%)[3].
- Clinical and experimental evidence shows that estrogen, the main premenopausal ovarian hormone, promotes metastases of TNBC, through their action on the tumor microenvironment.
- We have shown that reactive astrocytes in clinical and experimental brain metastasis models express estrogen-receptors (ER)[4].

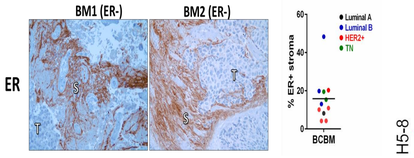


Figure 1. *Clinical specimens of b*reast cancer brain metastases stained for ERα. S= stroma, T= tumor. Graph shows quantification of ERα+ stroma in a cohort of brain metastasis according to molecular subtypes.

- The activity of aromatase (the enzyme that synthesizes estradiol, E2) is high in specific regions of the brain in females and in males. The brain of premenopausal women show areas of high aromatase activity[5].
- Brain metastases induce glial activation and are surrounded by aromatase-expressing astrocytes (Figure 2).
- Astrocytes play key roles in neuroinflammation and E2 decreases neuroinflammatory function of astrocytes.

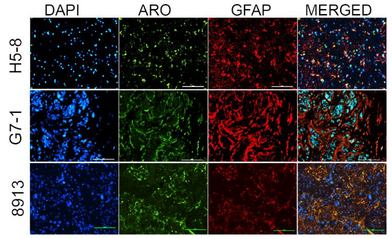


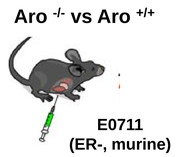
Figure 2. Breast cancer brain metastases were co-stained with aromatase (ARO, Green) and GFAP (Astrocytes, red). 20X images. Bars represent 100 µm.

HYPOTHESIS

E2 represses the neuroinflammatory function of ER+ astrocytes, dampening immune-surveillance and promoting tumor cell survival in early brain metastatic colonization

RESULTS

Non-ovarian aromatase promotes brain metastatic colonization of ER- BC



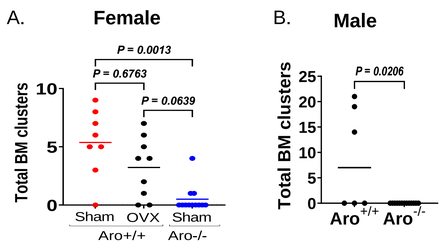


Figure 3. Murine breast cancer cells unresponsive to the mitogenic role of E2 in vitro (ER¯ E0711 cells) were injected intracardially in A) ovariectomized (OVX) or sham-operated (Sham) WT (Aro+/+) or sham-operated Aro KO (Aro-/-) female mice and brain metastasis were quantified 15 days later, or B) male Aro+/+ vs Aro-/- mice.

RESULTS

Astrocytes become reactive and express ER and aromatase since early stages of metastatic colonization

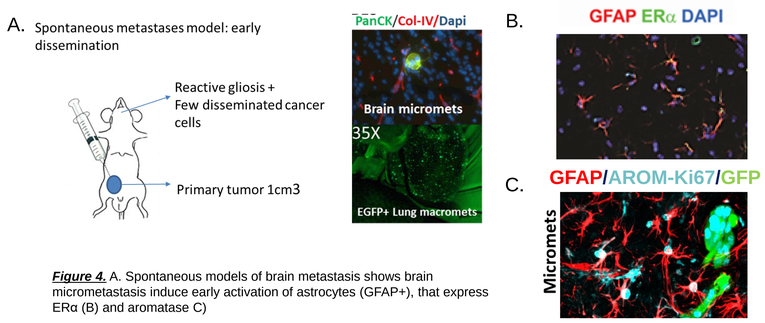


Figure 4. A. Spontaneous models of brain metastasis shows brain micrometastasis induce early activation of astrocytes (GFAP+), that express ERα (B) and aromatase C)

Aromatase inhibitors block E2 synthesis in astrocytes *in vitro*

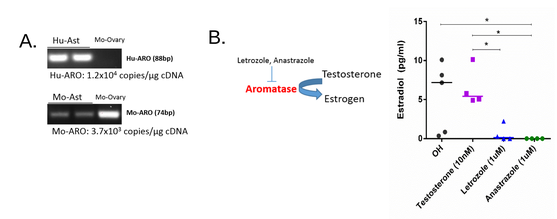
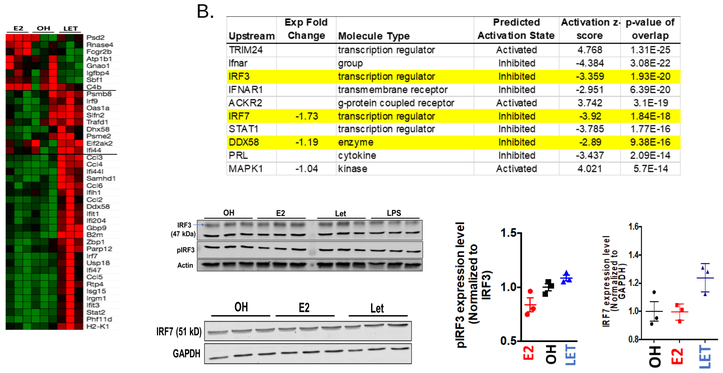


Figure 5. **A**. Human and mouse astrocytes express aromatase in vitro. B. Mass spectrometry shows aromatase inhibition blocks E2 synthesis by astrocytes in vitro.

Aromatase inhibitors upregulate IRF3/7 signaling in astrocytes *in vitro*



Upstream	Exp Fold Change	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap
TRIM24		transcription regulator	Activated	4.768	1.31E-25
Ifnar		group	Inhibited	-4.384	3.08E-22
IRF3		transcription regulator	Inhibited	-3.359	1.93E-20
IFNAR1		transmembrane receptor	Inhibited	-2.951	6.39E-20
ACKR2		g-protein coupled receptor	Activated	3.742	3.1E-19
IRF7	-1.73	transcription regulator	Inhibited	-3.92	1.84E-18
STAT1		transcription regulator	Inhibited	-3.785	1.77E-16
DDX58	-1.19	enzyme	Inhibited	-2.89	9.38E-16
PRL		cytokine	Inhibited	-3.437	2.09E-14
MAPK1	-1.04	kinase	Activated	4.021	5.7E-14

Figure 6. **A.** Mouse astrocytes were treated with 10nM E2, vehicle or 100nM Letrozole for 24hr (n=3 per group), and RNA expression analyzed using RNAseq. A. Heat map shows differentially expressed genes in one of the most upregulated pathways (IRF3). B. Upstream regulator analysis of differentially expressed genes showed that E2 represses a program of pro-inflammatory chemokines in the Interferon-Regulatory factor-3 and 7 pathways (IRF-3, P=1.93 x 10-20; IRF-7 P=1.84.93 x 10-18); and activates the transcriptional coactivator TRIM24 (P=1.31 x 10^25). C. Western blot confirmed repression of IRF-3 activation by E2 and upregulation of IRF-7 levels in letrozole-treated reactive astrocytes in vitro.

RESULTS

Conditioned media from letrozole-treated astrocyte increases M1/M2 polarization markers in primary microglia

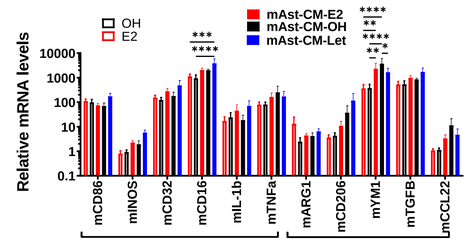
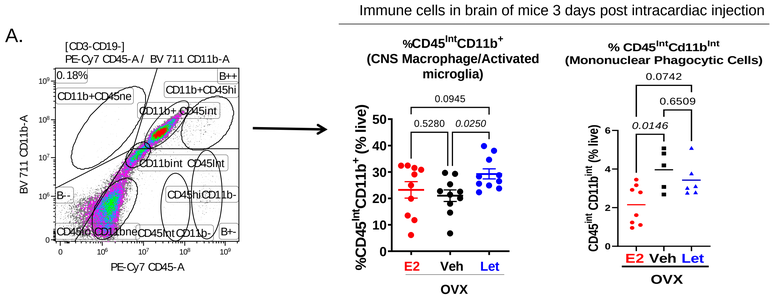


Figure 7. qRT-PCR for M1 and M2 microglial polarization and activation markers in primary microglia treated with vehicle (OH), E2 (10nM), or conditioned media from astrocytes (mAst-CM) treated with E2, OH or Letrozole for 24hr. Graphs shows summarized relative expression from 3-4 independent experiments.

E2 represses early microglia/CNS macrophage activation in the brain TME to promote survival of disseminated cancer cells



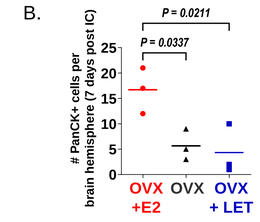
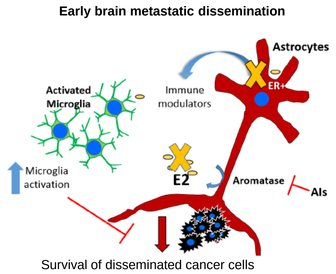


Figure 8. E0771 GFP-luc cells were injected in ovariectomized (OVX) C57Bl6 mice supplemented with 17-β-estradiol (E2), placebo (Veh) or letrozole (Let). Three days after injection, full brains were isolated and immune cells isolated and analyzed by flow cytometry. A. CD45IntCd11bint and CD45IntCD11b+ populations depicted on the right. B. Quantification of parenchymal PanCK+ cancer cells in OVX mice supplemented with E2, veh or Letrozole and injected with 231Br cells, 7 days post IC injection.

CONCLUSIONS

- Aromatase expressing astrocytes accompany brain metastasis progression.
- E2 (ovarian and peripheral) suppresses Type I-IFN anti-tumor responses via IRF3/7 activation in ER+ astrocytes, that in a paracri manner induced microglial activation.
- E2 does not promote direct classical M1 vs M2 microglial activation vitro, but conditioned media from letrozole-treated astrocytes increases expression of M1 polarization markers in microglia.
- These data support the notion that E2 represses immune-surveillance ir the early brain metastatic niche in favour of brain metastatic colonizatior and that aromatase inhibitors may reactivate innate anti-tumoral respons at early stages of metastatic dissemination.



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DISCLOSURES

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