THE ROLE OF CASPASE-1 IN TRIPLE NEGATIVE BREAST CANCER AND THE TUMOR MICROENVIRONMENT

Weiyue Zheng1, Wanda Marini (SSTP)1,2, Kiichi Murakami1, Pamela Ohashi1,3,4, Michael Reedijk1,2,4*

1Campbell Family Institute for Cancer Research, Ontario Cancer Institute, Toronto, Ontario, Canada, 2Department of Surgical Oncology, Princess Margaret Cancer Centre, University Health Network, Toronto, Ontario, Canada. 3Department of Immunology, University of Toronto, Toronto, Ontario, Canada. 4Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada

Hypothesis and Purpose

Breast cancer is the most common malignancy in women world-wide. In Canada, 26,900 new cases were diagnosed in 2019, accounting for 25% of all new cancer cases in women1. Breast cancer is a highly heterogeneous disease and clinical treatment is based on the expression pattern of specific hormone receptors (estrogen receptor (ER), progesterone receptor (PR), and HER2). While the majority of clinical breast cancers are ER and PR positive, also referred to as luminal breast cancer, triple negative breast cancer (TNBC), which lacks the expression of ER, PR and HER2, accounts for 10-15% of all breast cancers2.

Although TNBC represents the minority of all breast cancers, it represents a disproportionate number of breast cancer deaths due to its aggressive nature and high recurrence rate. Understanding what drives complex cell-cell interactions within the tumor microenvironment (TME) in TNBC is critical to developing new immunotherapeutic strategies in this disease. Previously, we demonstrated that in TNBC the Notch developmental signaling pathway is highly activated and directly drives expression of the genes encoding the inflammatory cytokines CCL2 and IL1β. The recruitment of these cytokines thereby promotes the recruitment of pro-tumoral tumor-associated macrophages (TAM) to the tumor microenvironment (TME)3. Here we report that TNBC are uniquely capable of IL1β secretion due to elevated expression of Caspase-1, a key component of the inflammasome required for cytokine maturation.

Methods

We analyzed expression arrays of breast cancer cell lines and primary tumors to demonstrate an association between the TNBC and Caspase-1 expression. Breast cancer cell lines were used to elucidate the mechanism of Caspase-1 hyper-expression in TNBC, and mouse models of TNBC confirmed the critical role of this mechanism in macrophage recruitment.

Results

Caspase-1 expression is inversely correlated with estrogen receptor expression and outcome in breast cancer

In previous work, we demonstrated that TNBC is associated with Notch activation, which drives the expression CCL2 and IL1β, and infiltration of pro-tumoral TAMs3. In that study we also showed that Caspase-1, which is required for IL1β maturation, is upregulated in TNBC in a Notch-independent fashion. Interrogation of public data set confirmed that RNA expression of pro-Caspase-1 was significantly higher in cell lines4 and primary tumors5 of the TNBC/ERα- subtype compared to the luminal/ERα+ subtype (Fig 1A, 1B). We observed that down-regulation of ERα in luminal breast cancer cell lines increased both Caspase-1 mRNA and protein expression (Fig 1C). The inverse relationship between ERα and caspase-1 expression was confirmed in a luminal
Breast cancer cell line that became tamoxifen-resistant and had lost the ability to express ERα (Fig 1D).

**Estrogen receptor regulates Caspase-1 expression through ETS1**

The transcription factor ETS Proto-Oncogene 1 (ETS1) is known to directly bind to the Caspase-1 promoter and regulate Caspase-1 expression\(^6\). ERα can sequester ETS1 and directly inhibit its binding ability and transcription activation\(^7\). Furthermore, in ERα positive breast cancer cells, ETS1, ER and nuclear factor P160 can form a complex to activate ER response genes \(^8\).

Based on these findings, we hypothesized that, in luminal breast cancer cells, ERα binds to and sequesters ETS1, thereby preventing its ability to bind to, and activate the Caspase-1 promoter. In support of this hypothesis, we found that Caspase-1 expression was tightly-controlled by the ratio of ETS1 to ERα (Fig 2A). Concomitant ETS1 over-expression and ERα knock-down in luminal breast cancer cells favoured Caspase-1 expression. TMX2-28 cells, where the ERα:ETS1 ratio was significantly reduced compared to wild-type MCF7, showed similar Caspase-1 over-expression. Conversely, ERα over-expression or ETS1 knock-down, decreased Caspase-1 expression (Fig 2B). A chromatin immunoprecipitation (CHIP) assay was performed to demonstrate that ETS1 binding to the Caspase-1 promoter was dependent on the ERα:ETS1 ratio. (Fig 2C). Overall, these findings indicate that the balance of ERα/ETS1 is critical for Caspase-1 expression (Fig 2D).

**Loss of Caspase-1 affects tumor growth and the immune cell infiltrates in vivo**

To further understand the role of Caspase-1 in vivo, we used a murine TNBC cell line derived from mammary tumors of K14-cre, BRCA1\(^{fl/fl}\), and p53\(^{fl/fl}\) (KBP) mice\(^9\). To confirm the association between ERα and Caspase-1 in mouse mammary tumor cells, transient transfection using serial quantities of a mouse ERα plasmid was performed. As seen in human cells and validating the use of the KBP model, Caspase-1 expression was inversely related to ERα expression in a dose-dependent manner (Fig 3A). As expected, CRISPR-mediated Caspase-1 KO resulted in reduced secreted IL1β levels both in vitro and in vivo (Fig 3B). Importantly, Caspase-1 KO resulted in reduced tumor growth (Fig 3C), and reduced macrophage infiltration (Fig 3D). Treating KBP allografts with colchicine, an inflammasome and Caspase-1 inhibitor, resulted in decreased tumor weight and decreased macrophage infiltration (Fig 3E).

**Conclusion**

We conclude that the lack of ERα in TNBC promotes ETS1-dependent Caspase-1 expression, allowing IL1β maturation, macrophage recruitment and tumor progression. Our data provides new insights into the biology of TNBC, illuminating novel potential immunotherapeutic strategies that target Caspase-1 expression.
Figure 1 Caspase-1 expression is inversely correlated with estrogen receptor expression and outcome in breast cancer

(A) mRNA level of Caspase-1 in 22 human breast cancer cell lines classified into two groups based on the subtype (luminal vs basal) (mean s.d., two-tailed, unpaired t-test with equal variances *: p<0.05). (B) mRNA level of Caspase-1 in 2508 breast tumor samples grouped either by subtype or by ERα expression (mean s.d., two-tailed, unpaired t-test with equal variances ****: p<0.0001) (C) Caspase-1 mRNA level in ERα positive MCF7 and T47D cell lines with ERα transient knockdown by siRNA (left) (n=3 biologically independent experiments, mean s.d., two-tailed, unpaired t-test with equal variances, *: p<0.05) and protein level in the MCF7 cell line (right). (D) Immunoblot of ERα and Caspase-1 in four tamoxifen-resistant MCF7 cell lines (left), and Caspase-1 mRNA level in the ERα- deficient TMX-28 cell line (right). Note: ERα loss results in Caspase 1 up-regulation in TMX2-28. n=3 biologically independent experiments (mean s.d., two-tailed, unpaired t-test with equal variances, ****: p<0.001).
Figure 2 Estrogen receptor regulates Caspase-1 expression through ETS1

(A) Real-time PCR and immunoblot analysis of ERα, ETS1 and Caspase-1 in MCF7 cells overexpressing ETS1, with and without ERα siRNA knockdown, and in TMX2-28 cells. For mRNA data, n=3 biologically independent experiments (mean s.d., two-tailed, unpaired t-test with equal variances, *: p<0.05, ***: p<0.001, ****: p<0.0001; N.S.=not significant). (B). Immunoblot of ETS1 and Caspase-1 in the TNBC cell line, MDA-MB-231, with either ERα over-expression or ETS1 knock-down. (C) Schematic of ChIP assay (left panel). Specific primers that amplify the ETS1 binding site on the Caspase-1 promoter were used for real-time PCR quantitation of ETS1 binding. Real-time PCR quantitation of the Caspase-1 promoter in wild-type and ETS1-overexpressing MCF7 cells, with and without ERα siRNA knockdown (middle panel), and in MDA-MB-231 cells with or without ERα over-expression (right panel) n=3 biologically independent experiments (mean s.d., two-tailed, unpaired t-test with equal variances, *: p<0.05, ***: p<0.001). (D) Table demonstrating the putative association between ETS1/ERα ratio, Caspase-1 expression and breast cancer subtype.
Figure 3 Loss of Caspase-1 affects tumor growth and immune cell infiltrates in vivo

(A) Serial amounts (0ug, 1.5ug, 3ug) of a mouse ERα-expressing plasmid was transiently transfected into KBP cells. mRNA expression and protein levels of Caspase-1 and ERα were analyzed via real time PCR (left) and western blot (right). (B) ELISA detection of cleaved IL1β in both wild-type and Caspase-1 KO KBP cells in vitro, as well as wild-type and Caspase-1 KO KBP allografts in vivo. (C) Growth curves and tumor weights of KBP wild-type and KBP Caspase-1 KO allografts. n=4 individual mice (mean s.d., linear regression for growth curves, *: p<0.05; two-tailed, unpaired t-test with equal variances for end point tumor weights, *: p<0.05). (D) Representative image for IHC staining of mouse macrophage marker F4/80 (brown staining) and FACs quantification of macrophage infiltration (CD11b and F4/80 double positive) for both KBP wild-type and KBP Caspase-1 KO allografts. Both IHC and FACs were performed on the same tumor. (E) KBP allografts treated with vehicle control or colchicine; (left) tumor weights, (right) flow cytometry data showing the percentage of macrophages (CD11b and F4/80 double positive) within the CD45 positive cell population. (n=4 individual mice, mean s.d., two-tailed, unpaired t-test with equal variances, *: p<0.05)
References


This project has taken place from August 2017 to present, with all the work performed in Dr. Michael Reedijk’s lab at the Princess Margaret Cancer Center. The supervisor of this project is Dr. Michael Reedijk. Weiyue Zheng is the major contributor of the project. Wanda Marini performed analysis of the cell line database and human breast tumor database. She also performed all of the statistical analysis. Kiichi Murakami performed all of the FACs experiments and was involved in the analysis of the FACs data. Pamela Ohashi is a collaborator PI.