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RESEARCH ARTICLE

Vaccine-induced T-helper-1 and endocrine therapy in an estrogen receptor alpha low breast cancer model

Denise L. Cecil*, Ekram Gad, Lauren Corulli, Kevin Potts and Mary L. Disis

UW Medicine Cancer Vaccine Institute, University of Washington, Seattle, WA.

*dcecil@uw.edu

ABSTRACT

The standard of care options for patients with triple negative breast cancer is limited to primarily surgery and chemotherapy. Recent evidence has demonstrated that combining immune therapeutic strategies with standard therapies increased efficacy in clinical trials and mouse models of breast cancer. Endocrine therapy, such as tamoxifen, has traditionally only been used to treat estrogen receptor positive breast cancer, but results of early tamoxifen clinical trials have revealed increased clinical activity in estrogen receptor-negative/low tumors. The goal of this study was to determine if combination immune therapy and endocrine therapy could inhibit tumor growth in an estrogen receptor-low mouse model. We observed that immunization targeting the tumor antigen insulin-like growth factor receptor-1 inhibited tumor growth dependent on interferon-gamma-secreting $CD4^+$ T-cells. Interferon-gamma upregulated expression of the tumor suppressor phosphatase and tensin homolog *in vitro*. Vaccination induced sensitivity to treatment with either tamoxifen or anastrozole and the combination therapy was more effective than any single agent alone. Our study suggests that strategies incorporating antigen-specific type I T cells in conjunction with non-standard endocrine therapy regimens can improve outcomes in patients with triple negative breast cancer.

Introduction

Several targeted treatment options are available for patients whose tumors express the estrogen receptor (ER), progesterone receptor or HER2[1]. However, for the approximately 15% of women whose tumors lack the expression of these receptors, those with triple negative breast cancer (TNBC), the standard of care is limited to largely surgery and chemotherapy[1]. Newer therapeutic strategies, such as immunotherapy, have been approved as treatment options in select TNBC patients[2]. Furthermore, blockade of the PD-1/PD-L1 axis in conjunction with standard chemotherapy has resulted in significantly improved complete response rates compared to cytotoxic therapy alone in patients with locally advanced or metastatic TNBC[3]. Standard and immune therapy combination regimens have also been reported in breast cancer mouse models[4]. Vaccine-induced interferon gamma (IFN-γ) derived from T-helper 1 (Th1) cells upregulated the suppressor of cytokine signaling-1 in tumor cells, resulting in the signaling inhibition of several growth factor receptors in oncogene addiction pathways. Cancer was completely eradicated when mice were treated with biological or cytotoxic therapy after vaccination[4]. Combining immune and standard therapies may lead to approaches that previously had no or low activity as single agents.

Historically, endocrine therapy using selective estrogen receptor modulators (SERMs), selective ER degraders (SERDs), and aromatase inhibitors (AI) have only been used to treat ER+ breast cancer due to the understanding that estrogen most classically exerts its effects through estrogen receptor alpha (ERα). It is known that estrogen-bound ERα enters the nucleus and binds with estrogen-responsive elements on the promoters of target genes resulting in the ability to promote the transcription of hundreds of genes involved in cell proliferation and differentiation[5]. However, the SERM, tamoxifen, has shown some clinical activity in ER-negative/low tumors. A meta-analysis evaluating tamoxifen responses in 133 clinical trials worldwide in 5,366 ER-negative/low patients, revealed a significant recurrence free survival rate of 13±4% and a significant overall survival rate of 11±5%, which was not dissimilar to the rate observed in ER+ patients (21±3%)[6].

The transcriptional processes activated by ERα in response to estrogen can also be facilitated by alternative receptors, such as ERβ and GPER1, in ER-negative/low tumors[7] and estrogen-induced activity through these alternative receptors can be modulated by tamoxifen[8-10]. Considering that vaccine-induced Th1 improved the efficacy of standard therapies[4], we questioned whether active immunization could synergize with standard endocrine therapies to control tumor growth in an ER-low model of mammary cancer.

Materials and Methods

Animal model and study design. Animal care and use were within institutional guidelines. The C3(1)-Tag [strain name: FVB-Tg(C3-1-TAg)cJeg/Jeg] basal breast cancer model expresses low levels of ER[11]. C3(1)-Tag male mice (provided by Dr. Jeff Green, NCI) were maintained by breeding to FVB/nJ parental females (Jackson Laboratory, strain #001800). A power analysis was used to

detect a difference in tumor volume and 5 mice per group was determined to provide 80% power to detect a significant pairwise difference at the two-sided alpha level of 0.05. Mice were randomly assigned to the different treatment groups before the start of the experiment. The investigators and animal caretakers were not blinded to the treatment groups. Studies were terminated when the volume of vaccinated group was statistically significantly less than the control for at least two measurements.

Immunization, treatment, and tumor growth. Mice were subcutaneously immunized three times with a mixture of 50 µg each IGF-IR peptide (p1166-1182, p1212-1226 and p1302-1325) in complete/incomplete Freund's adjuvant (Sigma) at 10-day intervals, or with adjuvant alone (control). The mouse mammary tumor cell line, M6, derived from spontaneous mammary tumors from C3(1)-Tag mice was authenticated before use and verified to express the SV40 antigen by Western blot and the estrogen receptor by RT-PCR. For tumor challenge, 0.5 x 10^6 cells were implanted into the flank two weeks after the third vaccine. Tumors were measured every two to three days with Vernier calipers, and tumor volume was calculated as the product of length x width x height x 0.5236. Treatment with tamoxifen (250 µg) or anastrozole (10 µg) was administered subcutaneously five times per week for the duration of the study. Tumor growth is presented as mean tumor volume (mm^3±SEM).

Measurement of antigen specific T-cell responses. Murine splenic cells were evaluated by IFN-γ ELISPOT[4]. Spleen cells were plated, 2x10^5 per well (5 replicates), with IGF-IR peptides or HIV Gag p52 (all 10 µg/mL) (CPC Scientific). Positive responses were defined as $p<0.05$ between the means of experimental and no antigen wells and reported as corrected spots/well (CSPW; experimental-no antigen control spots) ±SEM.

in vivo cell depletion and cytokine neutralization. Depletion of CD4+ and CD8+ T-cells was performed as published (>95% depletion)[4]. Cytokine neutralization was performed by intraperitoneal injection of anti-IFN-γ (100 µg; clone 37895; R and D Systems) or rat IgG2a isotype (clone 54447; R and D Systems) given four and two days prior to and on the same day as tumor inoculation, then repeated once weekly until study end[4]. All experiments were repeated twice with similar results.

Cell analysis by western blot and qRT-PCR. For the Western blot, M6 were stimulated with 100 ng/ml recombinant mouse IFN-γ (R & D Systems) and cultured in media/1% FBS for 18 hours followed by Western blotting using 25 µg cell lysates.[4] Antibodies used were tubulin and PTEN (1 µg/ml; Cell Signaling Technology). RNA was isolated using the RNAqueous-4PCR (Life Technologies) kit according to manufacturer's instructions. RNA quantity was determined with a Qubit Fluorometer. cDNA was synthesized from 100 pg of RNA using the SuperScript III RT (Life Technologies) kit according to the manufacturer's instructions then quantified. ERα, ERβ, GPER1, and ERRα expression was assessed via TaqMan (ABI 7900HT) Real time PCR using 50 ng of cDNA and 1 pg of each TaqMan Gene Expression Array (Life Technologies).

Statistical analysis. The unpaired, two-tailed Student's t test or ANOVA was used to

evaluate differences. A p value of <0.05 was considered significant (GraphPad Software, Prism v.9).

Results

T-helper 1 cells, elicited by vaccination, inhibited the growth of breast cancer via interferon-gamma secretion. High magnitude antigen-specific IFN-γ-secreting T-cells were generated when C3(1)-Tag mice were immunized with an IGF-IR peptide vaccine (mean, 908±181 cSPW per 10^6 splenocytes) as compared to adjuvant alone ($p<0.001$; Fig. 1A). Immunization inhibited M6 implanted tumor growth by 58% as compared to controls. ($p<0.0001$; Fig. 1B). M6 implanted tumor growth arrest following the IGF-IR vaccine was dependent on CD4+ T-cells. Tumor growth in mice treated with anti-CD8 after IGF-IR immunization was significantly inhibited as compared to the control ($p=0.009$), whereas tumor growth in mice depleted of $CD4^+$ T-cells was similar to control ($p=0.926$; Fig. 1B). Selectively neutralizing IFN-γ in a vaccinated luminal B breast cancer model prior to tumor challenge abrogated the anti-tumor activity of immunization[4], which we also observed here, in the C3(1)-Tag model. The average tumor volume in IFN-γ-neutralized mice tended to be larger than vaccinated mice treated with control IgG ($p=0.08$; Fig. 1C). Phosphatase and tensin homolog (PTEN) protein expression in the M6 cell line was increased after IFN-γ treatment (Fig. 1D).

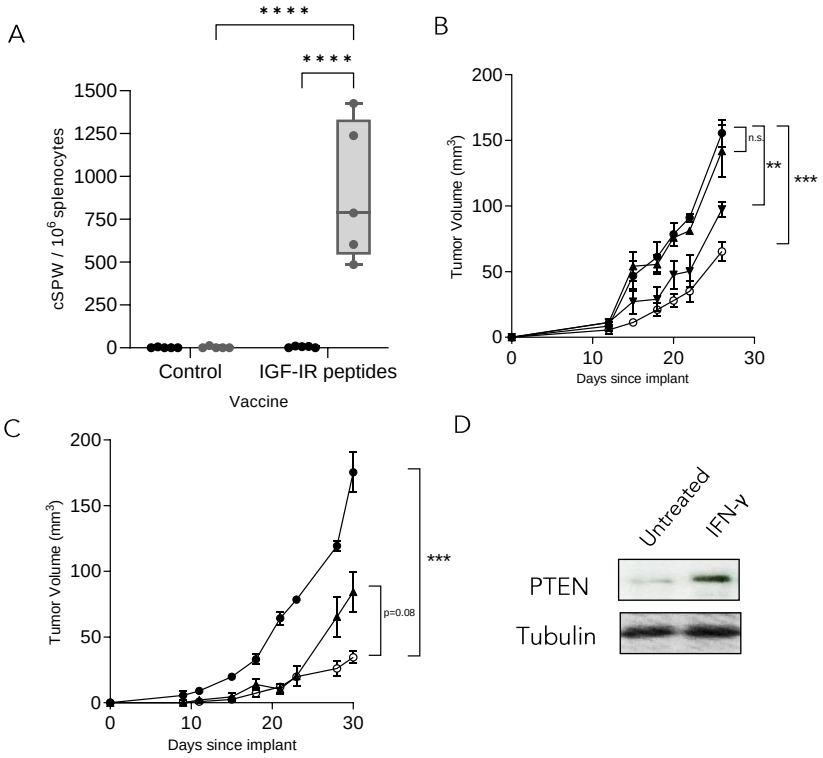

Figure 1. T-helper 1 cells, elicited by vaccination, inhibited the growth of breast cancer via interferon-gamma secretion. (A) Corrected IFN-γ spots per well (cSPW) per 10^6 splenocytes after mice were immunized with adjuvant only (control) or IGF-IR peptides and splenocytes stimulated with an irrelevant peptide (black bars) or the vaccinating peptide pool (gray bars) presented as box and whisker plots with horizontal line at median and whiskers at minimum and maximum showing all points. (B) Mean tumor volume (±SEM) of control mice (●) or mice immunized with an IGF-IR vaccine and treated with control IgG (○), anti-CD4 (▲) or anti-CD8 (▼). (C) Mean tumor volume (± SEM) of control mice (●) or mice immunized with an IGF-IR vaccine and treated with control IgG (○) or anti-IFN-g (▲). n=5 mice/group; *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001. (D) Western blot of non-phospho PTEN in the M6 tumor cell line after in vitro stimulation with IFN-γ.

Vaccination in combination with endocrine therapy inhibits tumor growth in an estrogen receptor negative breast cancer model. Genetic analysis of the M6 tumor cell line confirmed low expression of ERα. There was a 5-fold increase in ERβ, a 78-fold increase in GPER1 and a 2,418-fold increase in ERRα relative to ERα (Fig. 2A). Given the expression of alternative molecules that could be involved in mediating estrogen signals,[7] we questioned if endocrine therapy such as tamoxifen or anastrozole could modulate tumor growth. Tamoxifen treatment *in vivo* did not inhibit M6 tumor growth as compared to the control (p=0.408; Fig. 2B). However, when tamoxifen and IGF-IR vaccine were combined, tumor was inhibited by 70% compared to tamoxifen therapy alone (p=0.005) or by 58% from the IGF-IR vaccine alone (p=0.011; Fig. 2B). Similarly, tumor growth after *in vivo* treatment with anastrozole was equivalent to control (p=0.259). Treatment with anastrozole concurrently with IGF-IR vaccination resulted in significant tumor inhibition as compared to anastrozole alone (85% reduction; p=0.003) or vaccination alone (73% reduction; p<0.0001; Fig. 2C).

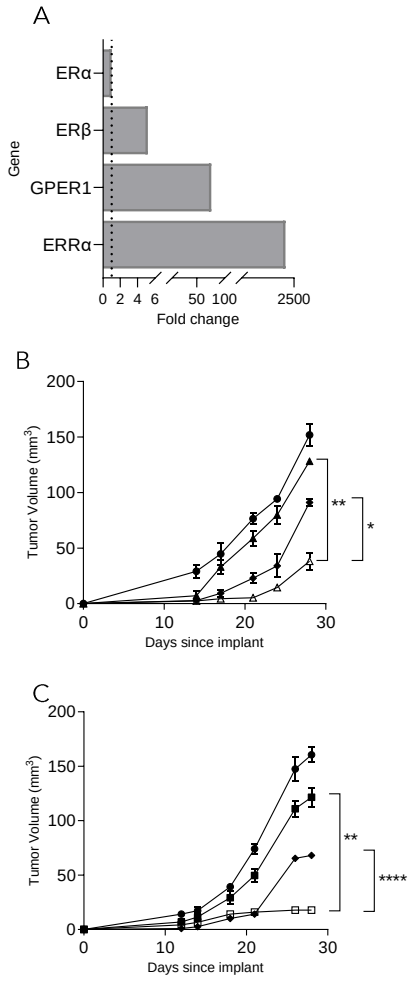

Figure 2. Vaccination in combination with endocrine therapy inhibits tumor growth in an estrogen receptor negative breast cancer model. (A) qRT-PCR for the indicated gene in the M6 tumor cell line. (B) Mean tumor volume (± SEM) of control mice (●) or mice treated with tamoxifen (▲) or immunized with an IGF-IR vaccine (♦) or treated with both tamoxifen and IGF-IR vaccine (Δ). (C) Mean tumor volume (± SEM) of control mice (●) or mice treated with anastrozole (■) or immunized with an IGF-IR vaccine (♦) or treated with both anastrozole and IGF-IR vaccine (□). n=5 mice/group; *p<0.05, **p<0.01, ****p<0.0001.

Discussion

In addition to mediating activation of specific immune effectors, tumor-specific Th1 cells can potentiate an anti-tumor immune response by upregulating molecules that directly inhibit proliferation signal pathways in the tumor, sensitizing treatment refractory tumors to standard therapies[4,12]. Data presented here demonstrate ER low breast cancer growth was significantly more controlled when endocrine therapy was combined with a Th1 vaccine, describing a novel role whereby TNBC could benefit from non-standard endocrine therapy.

Endocrine therapy, such as a SERM or AI, has been a fundamental treatment option for ER+ breast cancer for decades[13,14]. The SERM tamoxifen competes with estrogen to inhibit ER activity and the AI, anastrozole, blocks the synthesis of estrogen[15,16]. It was thought that endocrine therapy would be of little benefit to tumors lacking ER. ERβ, ERRα and GPER1 have been shown to mediate estrogen signals similarly as estrogen-bound ERα and expression of these alternative receptors is elevated in human ER-negative breast tumors[17-19], which we confirmed in our ER-low mouse model. ERβ is highly homologous to ERα and the two share a high degree of similarities in DNA-binding domains and in target gene regulation. It has been shown that ERβ expression can promote tumor growth in ER-negative breast cancer, irrespective of HER2/neu expression[20]. Expression of ERβ was a positive predictive factor of tamoxifen response in ER-negative breast cancer[8,9]. ERRα is an orphan nuclear receptor which can bind to similar genomic motifs as ERα, directing transcription of metabolic-related genes, supporting the energy requirements of proliferating cancer cells[21]. Pharmacological downregulation of ERRα activity decreased cell proliferation, tumorigenicity and reduced the cancer stem cell like phenotype in ER-negative breast cancer[22-24]. ERRα expression in patients treated with tamoxifen was associated with increased metastasis-free survival[15]. GPER1 is a G protein-coupled receptor that binds estrogen with high affinity and can mediate estrogen-induced second messenger signaling[25]. Silencing of GPER1 in TNBC cell lines has been shown to inhibit signaling cascades associated with cell growth[26]. Unfortunately, tamoxifen also acts as an agonist upon binding to GPER1 and is involved in the development of tamoxifen resistance in breast cancer[10].

We demonstrated upregulation in PTEN *in vitro* after treatment with IFN-γ, which blocks the proliferative PI3K/AKT signaling pathway, as had been reported previously[4]. Activation of the PI3K/AKT pathway is also a cytosolic non-genomic effect of estrogen-bound ERβ, in addition to direct transcriptional regulation of proliferative signals, similarly to that induced by estrogen-bound ERα (Figure 3)[7]. In other studies, perturbations in the PI3K/AKT/PTEN signaling pathway have been shown to sensitize cell lines to tamoxifen therapy[27,28]. We observed high expression levels of ERRα in the basal breast cancer cell line. There is no direct interaction between tamoxifen and ERRα as has been described for tamoxifen and ERβ[29,30], so it is possible that the inhibition of proliferation via IFN-γ-mediated growth factor receptor blockade simply decreases the energy needs of the cell[21], reducing the transcriptional activity of ERRα, thus facilitating tamoxifen's other ER-

independent effects (Figure 3). GPER1 bound with estrogen or tamoxifen can transactivate EGFR, promoting activation of the PI3K/AKT signaling pathway[31]. Thus, IFN-γ induced SOCS1-binding to EGFR after vaccination could effectively block the tamoxifen-GPER1 pro-tumorigenic interaction leading to the tumor growth arrest as we have observed in this study (Figure 3)[4].

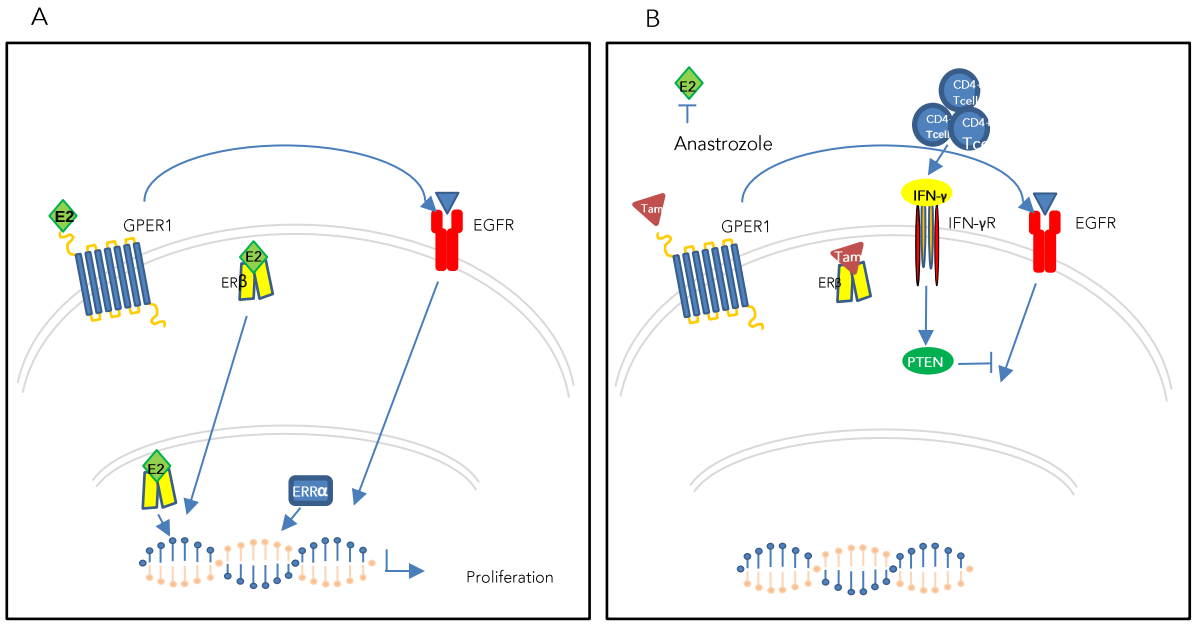

Figure 3. Potential mechanisms of action whereby generation of antigen-specific Th1 and endocrine therapy synergize to inhibit tumor growth in an ERα-low mouse model. (A) Estrogen (E2) can activate transcriptional programs involved in proliferation by directly binding to ERβ or transactivating EGFR by binding to GPER1. ERRα can also promote proliferation by binding to similar elements as estrogen-bound ERα. (B) Anastrozole blocks the formation of E2 and tamoxifen (Tam) outcompetes E2 for binding with ERβ, inhibiting transcription of proliferation-related genes. Th1-induced IFN-γ upregulates PTEN which can block proliferation signals transduced via direct activation or tamoxifen-bound GPER1 transactivation of EGFR. This signaling blockade subsequently decreases the energy needs of the cell, reducing the transcriptional activity of ERRα.

In conclusion, studies such as this demonstrate that integrating strategies that increase type I T cells in conjunction with standard well-established endocrine therapy regimens can increase therapeutic efficacy. These data provide evidence that targeted non-standard therapies in ER-negative/low tumors can be employed for improved outcomes in combination with immunotherapeutic approaches.

Conflict of Interest:

None

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