

Bexarotene Enhances Anti-Tumor Efficacy with Cytotoxic Chemotherapy and Increases CD8+ T cell Infiltrate in Breast Tumors

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Abstract

Background: Increased CD8+ T cells in the tumor has shown improved response to therapy and prognosis in breast cancer. Cytotoxic chemotherapies increase immune recognition of the tumor but the effect of chemotherapy on the tumor immune environment is a balance between immune activation and systemic immune suppression. The retinoic acid receptor agonist bexarotene previously was shown to increase intratumoral CD8+ T cells in TgMMTV-neu transgenic mouse tumors. We therefore determined whether bexarotene could enhance the anti-tumor efficacy of common breast cancer chemotherapies and whether the immune role of bexarotene was contributing to the anti-tumor benefit.

Methods: In two immune competent mouse mammary tumor models, TgMMTV-neu and C3(1)Tag mice, spontaneous tumors were treated with bexarotene and chemotherapy (doxorubicin and paclitaxel in both models, cyclophosphamide in TgMMTV-neu mice, and cisplatin in C3(1)Tag mice) and the tumors were evaluated for CD8 and CD4 T cells. Immune cell populations that express the RXRa receptor were evaluated in human peripheral blood mononuclear cells (PBMC) and the direct role of bexarotene on human dendritic cell populations was evaluated by flow cytometry.

Results: Bexarotene enhanced tumor growth inhibition with all chemotherapies in TgMMTV-neu mice, decreasing growth by 98.1% with doxorubicin, 88% with cyclophosphamide, and 89% with paclitaxel. In C3(1)Tag mice bexarotene and cisplatin decreased tumor growth by 99%. Bexarotene doubled the incidence of CD8+ T cells in the tumors of TgMMTV-neu mice and 5.7 fold increase in C3(1)Tag mice. RXRa receptors were expressed highest in human monocyte (mDC, 33.1±5.7%) and plasmacytic dendritic cells (pDCs 38.6±4.8%), and in 25±4.4% macrophages. Bexarotene activated type I mDCs and pDCs that released IL-1β and TNFα type I cytokines.

Conclusions: Bexarotene enhances the anti-tumor efficacy of cytotoxic chemotherapy in transgenic mouse mammary tumor models and increases intratumoral CD8+ T cells. Bexarotene directly activates type I mDCs and pDCs.

Keywords: Bexarotene, Dendritic cells, Breast cancer, Macrophages

Background

As a whole, breast tumors have fewer immune cells infiltrating the tumor than more immune active cancers including melanoma and lung cancer [1]. In breast cancer tumor infiltrating lymphocytes (TILs) analysis, the immune infiltrate is found mostly in the stroma surrounding the

tumor rather than infiltrating the tumor. In the aggressive triple negative breast cancer subtype, each decile increase in percent stromal TILs prior to therapy is associated with improved overall and disease free survival [2]. However, even in the triple negative subtype only 20% of tumors are immune high (>50% TILs) while the majority have low TIL infiltrate [3]. Therapies are needed to increase immune infiltrate

in most breast cancers to get similar prognostic benefit. Chemotherapy, which is cytotoxic to CD8+ T cells, can have multiple immunostimulatory roles that impact the tumor immune environment. These include decreasing regulatory T cells and regulatory cytokines, increasing immunogenic cell death, increasing immune recognition of the tumor, and activating anti-tumor innate immune stress responses [4,5]. Many chemotherapies have been shown to enhance the anti-tumor immune response and impact the tumor immune environment. Three of the chemotherapies used predominantly in breast cancer therapy have demonstrated improved immune function. Doxorubicin has been shown to deplete myeloid derived suppressor cells (MDSC) and enhance CD8 T cell infiltration in mice and increases T cell cytotoxicity and inflammatory genes in triple negative breast cancer patients [6–8]. Paclitaxel has been shown to shift macrophage profiles to M1 macrophages through TLR4 signaling in a triple negative mouse model [9]. Cyclophosphamide has been shown to increase regulatory T cell apoptosis in mouse models of breast cancer, but the immune function differs between low and high doses of cyclophosphamide [10–12]. These immune functions all have to be considered with the balance of systemic immune suppression from the cytotoxic effect of chemotherapy therefore finding additional therapies that can enhance anti-tumor immunity may improve the immune stimulatory properties and eventual clinical efficacy of chemotherapy.

The rexinoid family are vitamin A derivatives and a nuclear receptor family that homo- or heterodimerize on retinoic acid responsive elements as ligand-dependent transcription factors to regulate multiple cancer signaling pathways [13,14]. Rexinoids have been associated with multiple cell functions including proliferation, differentiation, modifying the immune system, and apoptosis [15,16]. Bexarotene is a synthetic rexinoid acid receptor agonist that has an FDA approved indication in cutaneous T cell lymphoma therapy [17]. Furthermore, it has efficacy in breast cancer, in 100 metastatic hormone receptor positive patients that were resistant to hormone therapy there was a clinical response in 20% of patients receiving bexarotene as monotherapy but the majority of responses were stable disease [18]. In mouse models, bexarotene was shown to have a role in decreasing proliferation by decreasing cyclin D1 activity through inducing the transcriptional repressor DEC2 [19]. Bexarotene was also shown to have an immune role, enhancing the efficacy of a HER2- IGF1R peptide vaccine by preventing invasive cancer in 87% of mice as compared to 67% of mice with vaccine alone (HR 18.1 95% CI 6.4 to 63.2, $p < 0.0001$). This was associated with an increased intratumoral CD8+ T cell infiltrate in the tumors that developed in mice treated with bexarotene alone as compared to adjuvant controls ($P = 0.015$) and in mice treated with bexarotene and the HER2-IGF1R peptide vaccine as compared to the vaccine alone ($P = 0.0014$) [21]. Similarly, the rexinoid receptor agonist MSU-42001 has been shown to

modulate CD8+ T cell expression and reduce tumor burden in a neurofibromatosis mouse cancer model with or without a MEK inhibitor [20]. We therefore wanted to determine if the addition of bexarotene increased intratumoral CD8+ T cells as compared to chemo alone.

Rexinoids have been previously shown to modulate the tumor immune environment, particularly impacting the innate immune response, in mouse mammary tumor models. The rexinoid acid receptor LG100268 showed to decrease MDSC and M2 (CD206 positive) macrophages in the MMTV-PyMT triple negative transgenic mouse mammary tumor model thus increasing the CD8/CD4 ratio in the tumor [22]. Activating antigen presenting cells (APCs) and the innate immune environment can induce a more anti-tumor immune environment allowing for more CD8+ T cell infiltration and enhancing the function of chemotherapy. This study was to evaluate whether bexarotene enhances the efficacy of chemotherapy and determine how bexarotene directly impacts the immune response in human immune cells.

Methods

Maintenance of the TgMMTV-neu mice

TgMMTV-neu mice were obtained from Jackson Laboratories and maintained under strict inbreeding conditions and the colony was re-tested for expression of the neu transgene annually. All mice were bred and maintained under specific pathogen free conditions based on the University of Washington institutional animal care and use committee (IACUC) protocol.

Tumor evaluation in the transgenic mice

Mice were enrolled into the study upon development of measurable, spontaneous, subcutaneous breast tumors. Mice were randomly allocated to treatment groups. Therapy started when the tumor reached $\sim 100 \text{ mm}^3$ and was continued for four weeks unless the tumors were ulcerated or tumor volume exceeded 10% of overall mouse body weight. Sample size was calculated as 5 per group based on the power calculation of mean difference of 2 with 2 sided $\alpha = 0.05$ with power of 80%. Mice were examined for tumor development, and tumor growth was measured three times per week from six weeks of age until sacrifice. Tumor volumes were calculated from raw measurements by $[(\text{length}) \times (\text{width}) \times (\text{depth}) \times (\pi/6)]$ and are presented as an average of the three weekly measurements. Multiple tumors were tracked independently. Tumor growth rates were calculated by determining the change in sum of tumor volumes between subsequent measurements.

Dosing and delivery of bexarotene and chemotherapy

The chemotherapies were given intravenously through the tail vein weekly. Doxorubicin was dosed at 5 mg/kg,

cyclophosphamide was dosed at 100 mg/kg, and Paclitaxel was dosed at 10 mg/kg infused weekly. Control mice had 50 μ L PBS infused weekly. Bexarotene was dissolved in sesame oil and given 5 days a week by oral gavage at 50 mg/kg and control mice were given 100 μ L of sesame oil 5 days per week by oral gavage.

Evaluation of the tumor immune infiltrates

At sacrifice, half of the largest measurable tumor was processed for flow cytometry. For flow cytometry evaluation, immune cells were isolated from the spleen through a 70 μ m cell strainer and in the tumor immune cells were enriched through FICOL gradient. Fresh cells were stained with a live/dead Pacific Blue fixable viability dye and PE CD3, PE Cy-5 CD4, and PE Cy-7 CD8. All antibodies were from ebioscience. These cells were analyzed by flow cytometry on a FACSCanto flow cytometer. Flow analysis was blinded to treatment arm. Flow cytometry gating strategy was shown in **Supplemental Figure 5**.

RXR expression in human PBMC

Human peripheral blood mononuclear cells (PBMC) from women without breast cancer (n=15) were used from the University of Washington Cancer Vaccine Institute repository (University of Washington Human Subject Division STUDY0001570). 2×10^6 cells were stained with the surface monoclonal antibodies and prepared for the intracellular RXR stains using the FOXP3 intracellular stain kit (ebioscience), fixed with 1.6% paraformaldehyde, measured on a BD FACS CANTO II flow cytometer, and evaluated on FlowJo software. RXR α expression was measured using a FITC RXR α monoclonal antibody (Millipore) in immune cell populations PE Cy7 CD3+ PECy5 CD8+ T-cells, PECy7 CD3+ PE CD4+ T-cells, PE CD56+ NK T-cells, PECy7 CD20+ B-cells, PECy5 CD11c+ APC HLADR+ PECy7 LIN- monocyctic dendritic cells, PE CD123+ APC HLADR+ PECy7 LIN- plasmocytic dendritic cells, PE CD14+ APC HLADR+ macrophages, PE CD11b+ PECy7 LIN- APC HLADR- monocyctic MDSC, and APC HLADR- PE CD14+ PECy5 CD33+ PE CD11b+ plasmocytic myeloid derived dendritic cells. All flow cytometry antibodies were from ebioscience (ThermoFisher Scientific). Live cell populations were selected using a Pacific Blue fixable viability dye (ebioscience). Fluorescence minus one controls were used in panel development. Flow cytometry gating strategy was shown in **Supplemental Figure 6**.

Activation of antigen presenting cells by bexarotene

PBMC from women without breast cancer (n=8) were used from the University of Washington Cancer Vaccine Institute repository. 1.5×10^6 human PBMC per sample were plated in 48 well tissue culture plates with 0, 5, 10, and 20 μ M of bexarotene diluted in RPMI T-cell media. As bexarotene needed 10% DMSO to go into solution, 0 μ M is RPMI with

10% DMSO. The bexarotene stock was 10 mM diluted into DMSO. The cells were incubated at 37°C for 48 hours and then collected, washed, and stained for FITC CD40 and PE CD11c+ APC HLADR+ PECy7 LIN- monocyctic dendritic cells, PE CD123+ APC HLADR+ PECy7 LIN- plasmocytic dendritic cells, and PE CD14+ APC HLADR+ macrophages. All antibodies were from ebioscience (ThermoFisher Scientific). Fluorescence minus one controls were used in panel development. The cells were stained with antibodies, fixed with 1.6% paraformaldehyde, measured on a BD FACS CANTO II flow cytometer, and evaluated on FlowJo software. Flow cytometry gating strategy was shown in **Supplemental Figure 7**.

Evaluation of cytokine release by bexarotene

Supernatant from the above 48 hour experiments were collected and immediately frozen at -80°C. Ten cytokines were evaluated by a multiplex magnetic fluorescent bead assay (Invitrogen, Carlsbad CA). The assay was read on a LiquidChip instrument (Qiagen, Valencia CA).

Evaluation of bexarotene in human dendritic cell maturation

Three independent human PBMC donors were evaluated. 100×10^6 cells were thawed in Iscove's modified Dulbecco's media (IMDM) supplemented with 10% FBS and selected for CD14+ monocytes using autoMACS (Miltenyi Biosciences, CA). The cells were then suspended in media that contained IL4 (Peprotech, 20 ng/mL) and GM-CSF (Peprotech, 50 ng/mL) and stored at 37°C at 5% CO₂ with media changed every 2 days. On day 6 immature dendritic cells were harvested and counted, and half the cells were maintained as immature dendritic cells with continued IL-4 and GM-CSF supplemented media until day 8. The other half of the cells were matured using IL-1 β (Peprotech, 25 ng/mL), TNF- α (Peprotech, 50 ng/mL), IFN- α (Peprotech, 3×10^3 IU/mL), IFN- γ (Peprotech, 100 ng/mL), Poly I:C (Peprotech, 20 μ g/mL), IL-2 (Peprotech, 5 ng/mL), and IL-7 (Peprotech, 10 ng/mL) [23,24]. All cells were harvested and evaluated at day 8 using the Cytoflex flow cytometer (Beckman Coulter). In flow cytometry, all cells were stained for far blue live/dead, CD56 BV510, HLA-DR BV605, CD123 BV650, CD16 BV785, CD33 PE-CF594, CD15 PerCP-Cy5.5, CD11c PE-Cy7, CD14 Alexa700, CD3 APC-H7, CD19 APC-H7, CD20 APC-H7, CD11b BV421, and CD40 PE (all antibodies BD Biosciences). Fluorescence minus one controls were used in panel development. Flow cytometry was run on the BD Fortessa and data was analyzed in FlowJo 10. Flow cytometry gating strategy was shown in **Supplemental Figure 7**.

Statistical analysis

Graphs, t-tests, and ANOVA comparisons were completed using GraphPad Prism v5.03 software. A One-Way repeated measures ANOVA with Tukey's post-test was used for

comparisons of tumor growth differences in mice and a two-Way ANOVA with Bonferroni's post-test was used for grouped comparisons. Significance was considered at $p < 0.05$ for all statistical tests.

Results

Bexarotene enhances the anti-tumor effect with chemotherapy in two mouse mammary tumor models

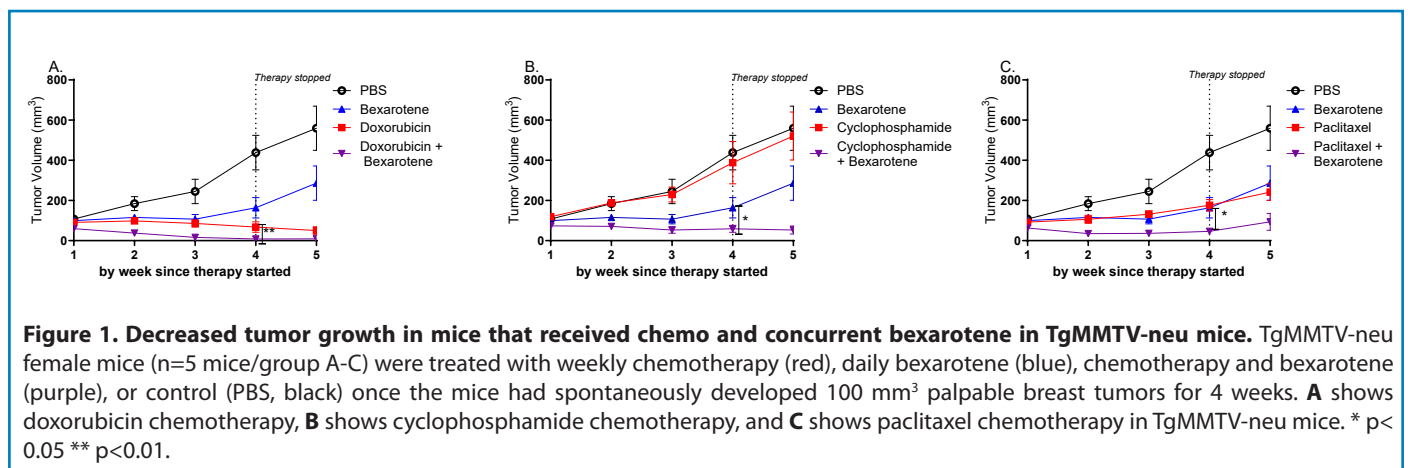
In TgMMTV-neu mice with spontaneous established tumors $\sim 100 \text{ mm}^3$ ($n=5$), the addition of bexarotene to doxorubicin, cyclophosphamide, or paclitaxel reduced tumors significantly more than the chemotherapies alone. Treating the mice with bexarotene for four weeks reduced tumor size by 65.9% compared to PBS treated mice ($p=0.003$, mean of bexarotene treated tumors were $149.6 \pm 41.8 \text{ mm}^3$ as compared to $438.3 \pm 86.2 \text{ mm}^3$ in PBS treated tumors). Doxorubicin reduced tumor size by 81.7% compared to PBS treated mice ($p=0.0005$, mean of doxorubicin treated tumors were $80.11 \pm 24.0 \text{ mm}^3$ compared to PBS) while mice treated with bexarotene and doxorubicin reduced tumor size by 98.1% ($p < 0.0001$ compared to PBS and $p=0.02$ compared to doxorubicin therapy alone, tumors were $8.2 \pm 2.4 \text{ mm}^3$, **Figure 1A**). While cyclophosphamide did not decrease tumor size as compared to PBS controls ($p=0.9$), cyclophosphamide and bexarotene decreased tumor growth by 87.7% ($p=0.009$ compared to PBS and $p=0.02$ compared to cyclophosphamide, mean tumor size was $59.3 \pm 17.8 \text{ mm}^3$, **Figure 1B**). Paclitaxel decreased TgMMTV-neu tumor growth by 57% as compared to PBS controls ($p=0.02$, mean size of tumor $176.1 \pm 28.2 \text{ mm}^3$) and bexarotene and paclitaxel further decreased tumor growth by 88.5% ($p=0.02$, mean size of tumor $50.4 \pm 5.6 \text{ mm}^3$, **Figure 1C**). Individual mouse growth curves for all treatment groups were shown in **Supplemental Figure 3**.

In C3(1) Tag mice with spontaneous tumors $\sim 50 \text{ mm}^3$, treatment with four weeks of bexarotene could inhibit tumor growth by 73.4% ($p=0.02$, mean size of PBS treated tumors

is $912.4 \pm 200.1 \text{ mm}^3$ and mean size of bexarotene treated tumors is $243.0 \pm 32.7 \text{ mm}^3$, **Supplemental Figure 1**). However, Bexarotene only enhanced tumor inhibition with cisplatin, cisplatin decreased tumor growth by 86.2% (mean tumor with cisplatin was $125.7 \pm 84.20 \text{ mm}^3$) while cisplatin and bexarotene decreased tumor growth by 98.6% ($p=0.02$, mean tumor with bexarotene and cisplatin was $12.20 \pm 6.0 \text{ mm}^3$) (**Supplemental Figure 1B**). Bexarotene, doxorubicin, and bexarotene with doxorubicin had similar tumor inhibition but bexarotene did not enhance tumor inhibition of doxorubicin (**Supplemental Figure 1A**). Paclitaxel and bexarotene did not further inhibit tumor growth as compared to bexarotene alone (**Supplemental Figure 1C**). Individual mouse growth curves for all treatment groups were shown in **Supplemental Figure 4**. As bexarotene function differed between chemotherapies and mouse models, we suspected that the role of bexarotene was beyond its impact on cell proliferation [19] and therefore wanted to evaluate whether the immune function of bexarotene may be contributing to the tumor inhibition.

Bexarotene increases CD8 T cells and decreases CD4 T cells in the tumors

To evaluate the impact of bexarotene on the tumor immune environment, we evaluated T cells in the tumor at 4 weeks of therapy by flow cytometry. In TgMMTV-neu mice, there was a significant increase in CD3+ CD8+ and a decrease in CD3+CD4+ populations in the tumor of mice treated with 4 weeks of bexarotene or any of the chemo as compared to PBS or oil controls. However, bexarotene with the chemotherapies did not either increase CD8+ T cells or decrease CD4+ T cells as compared to chemotherapy or bexarotene alone (**Figures 2A–2F**). Treatment with bexarotene doubled CD3+CD8+ T cells as compared to PBS ($p=0.02$ mean CD3+CD8+ T cells with PBS $21.0 \pm 6.4\%$ and mean CD3+CD8+ T cells with bexarotene $52.2 \pm 2.4\%$). However, while treatment with doxorubicin increased CD3+CD8+ T cells as compared to PBS (mean doxorubicin treated CD3+CD8+ T cells $69.7\% \pm 0.1$ ($p=0.01$), treatment with bexarotene and doxorubicin had significantly



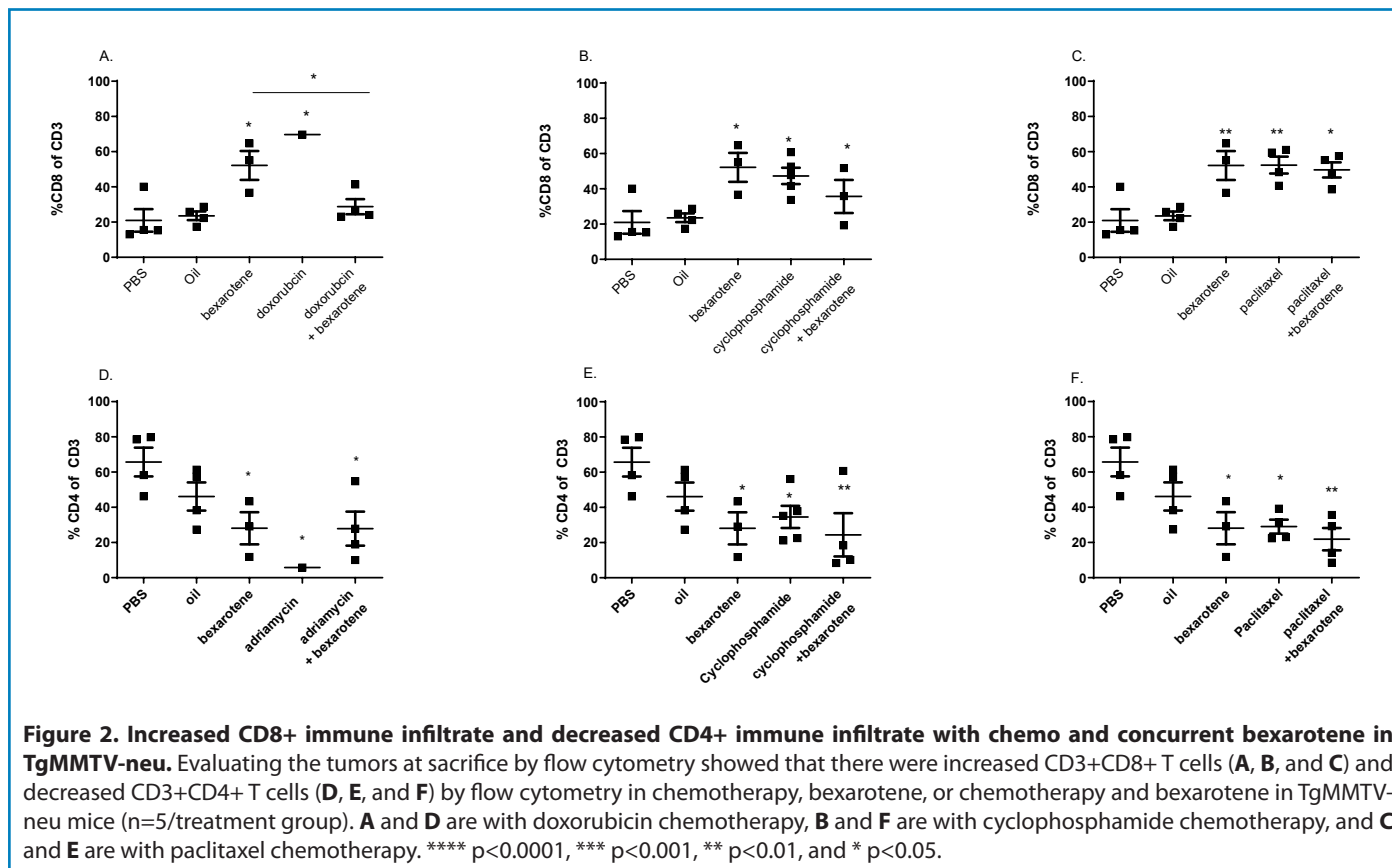
less CD8+ T cells than doxorubicin alone ($28.8 \pm 4.3\%$ CD3+CD8+ T cells, $p=0.03$, **Figure 2A**) despite improved tumor inhibition. Treatment with cyclophosphamide (CD3+CD8+ T cells $47.3 \pm 4.6\%$, $p=0.03$) and cyclophosphamide and bexarotene (CD3+CD8+ T cells $35.7 \pm 9.4\%$, $p=0.04$) both increased CD8+ T cells in the tumor but addition of bexarotene did not have more CD8+ T cells compared to cyclophosphamide alone (**Figure 2B**). Treating TgMMTV-neu mice with paclitaxel or paclitaxel and bexarotene increased CD3+CD8+ T cells but there was no increase with paclitaxel plus bexarotene as compared to paclitaxel alone ($52.4 \pm 4.3\%$ CD3+CD8+ T cells with paclitaxel, $p=0.005$ and $49.7 \pm 4.3\%$ CD3+CD8+ T cells with paclitaxel and bexarotene, $p=0.01$, **Figure 2C**). Similarly, both bexarotene and chemotherapy increased CD3+CD8+ T cells in the C3(1)Tag mouse tumors both there was only increase in CD8+ T cells with doxorubicin and bexarotene ($p=0.004$) but none of the other chemotherapies (**Supplemental Figure 2**).

While increasing CD8+ T cells, bexarotene, chemotherapy, and bexarotene with chemotherapy also decreased CD4+ T cells. In TgMMTV-neu tumors, there was a 3.7 fold decrease in CD3+CD4+ T cells when treated with bexarotene as compared to PBS controls ($20.5 \pm 8.7\%$ in tumors treated with bexarotene as compared to $65.7 \pm 8.2\%$ in PBS controls, $p=0.05$). Doxorubicin decreased CD3+CD4+ T cells in the tumor to $5.8 \pm 0.01\%$ ($p=0.05$) but the combination of doxorubicin

and bexarotene did not decrease CD3+CD4+ T cells more than doxorubicin alone to $27.9 \pm 9.7\%$ ($p=0.05$) (**Figure 2D**). Cyclophosphamide treated tumors had decreased CD3+CD4+ T cells in the tumor to $29.2 \pm 4.3\%$ ($p=0.01$) but the combination of cyclophosphamide and bexarotene decreased CD3+CD4+ T cells in the tumor to 12.3 ± 3.1 ($p=0.001$, **Figure 2E**). Treatment of tumors with paclitaxel decreased CD3+CD4+ T cells to $29.0 \pm 4.0\%$ ($p=0.02$) but the paclitaxel and bexarotene decreased CD3+CD4+ T cells in the tumor to $21.9 \pm 6.3\%$ ($p=0.004$, **Figure 2F**). Bexarotene enhances anti-tumor impact with chemotherapy, yet while it increases CD8+ T cells and decreases CD4+ T cell the impact is increased over chemotherapy alone therefore we wanted to better understand possible roles of bexarotene on the human immune system.

In human PBMC RXR receptors are highly expressed in dendritic cells and macrophages

As bexarotene could increase CD8+ infiltrate in tumors and had anti-tumor function with chemotherapy in the mouse, we wanted to determine the immune cell populations that had highest RXR α expression in human PBMC. RXR α had the highest expression in ~30% of human APCs, $24.9 \pm 4.4\%$ of macrophages (CD14+HLADR+), $38.6 \pm 4.8\%$ of plasmacytoid dendritic cells (pDC), and $33.1 \pm 5.7\%$ of monocytoïd dendritic



cells (mDC) (**Figure 3A**). The CD4+CD3+T cells had $4.5 \pm 1.1\%$ of the cells expressing RXR and CD8+CD3+ T cells had $3.1 \pm 0.6\%$ of the cells expressing RXR. NK cells (CD56+CD16+ cells) had $0.18 \pm 0.03\%$ cells expressing RXR, B cells (CD20+ cells) had $0.6 \pm 0.2\%$ cells expressing RXR, and granulocytic MDSC cells (LIN-CD14+CD33+HLADR-) had $0.05 \pm 0.02\%$ cells expressing RXR. Monocytic MDSC (LIN-CD11b+HLADR-CD33+ cells) had $5.9 \pm 2.2\%$ cells expressing RXR.

Increasing doses of bexarotene increase type I dendritic cells in human PMBC

As bexarotene was expressed in APCs, we wanted to determine how increasing doses of bexarotene could impact the APCs. Treating human PBMC with 0 μM , 5 μM , 10 μM , and 20 μM doses of bexarotene increased CD40+ activation of pDC and mDC but not macrophages (**Figures 3B–3D**). 20

μM of bexarotene increased activated mDC ($4.9 \pm 0.8\%$ LIN-CD11c+HLADR+CD40+ cells/LIN-CD11c+ cells with 20 μM bexarotene and $0.8 \pm 0.2\%$ with 0 μM bexarotene $p=0.01$) (**Figure 3B**). Both 10 μM and 20 μM of bexarotene increased CD40+ pDC activation as compared to 0 μM of bexarotene ($9.4 \pm 2.2\%$ LIN-CD11c+HLADR+CD40+ cells/LIN-CD11c+HLADR+ cells with 10 μM bexarotene $p=0.03$, $8.7 \pm 1.9\%$ LIN-CD11c+HLADR+CD40+ cells/LIN-CD11c+HLADR+ cells with 20 μM bexarotene $p=0.05$, and $3.1 \pm 1.2\%$ with 0 μM bexarotene) (**Figure 3C**). There was no significant increase in activated macrophages with 20 μM of bexarotene ($p=1.0$) (**Figure 3D**). We further evaluated the cytokines released with increasing doses of bexarotene. Evaluating 10 different cytokines, we found that there were increased type I cytokines IL-1 β ($p=0.04$ between 0 and 20 μM bexarotene **Figure 4A**) and TNF α ($p=0.03$ between 0 and 20 μM bexarotene **Figure 4B**) but not type II cytokines including IL-10 ($p=0.99$) and IL-4 ($p=0.97$) (**Figures 4C and 4D**).

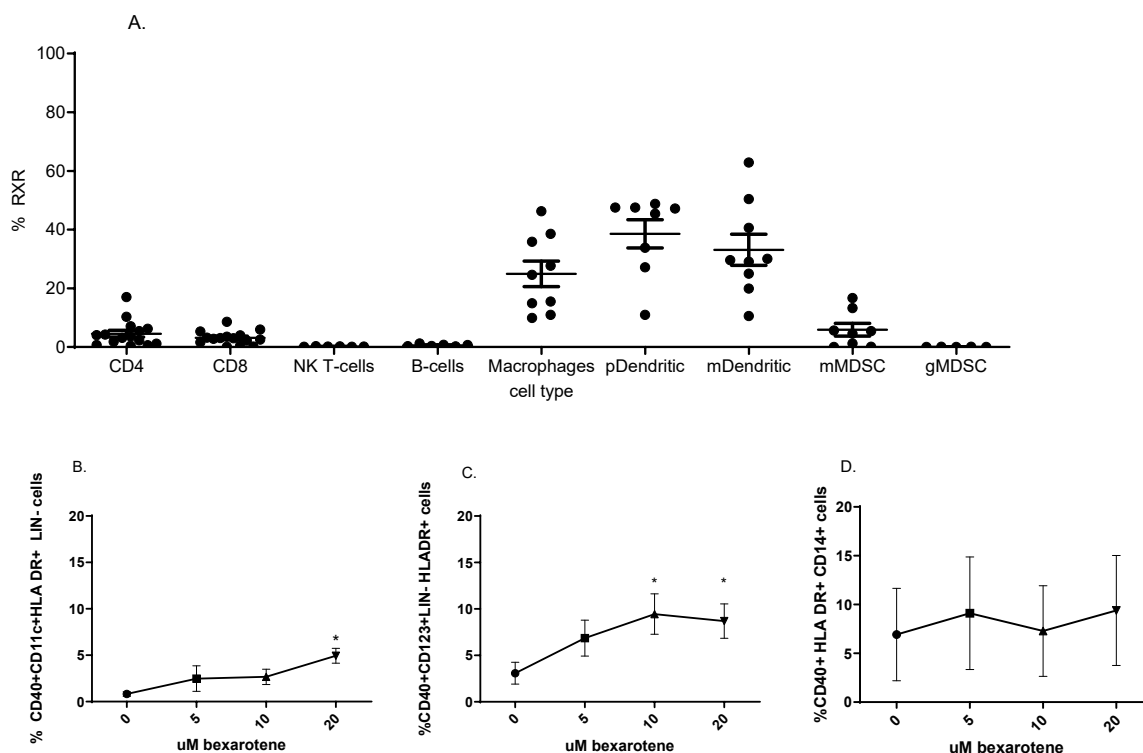


Figure 3. RXR is expressed primarily in antigen presenting cells in human PBMC and bexarotene increases CD40 expression in both monocytic and plasmacytic dendritic cells. 2×10^6 peripheral blood mononuclear cells from women without breast cancer ($n=15$) were evaluated for expression of the retinoid X receptor (RXR) by flow cytometry. (**A**) CD4+ T cells: RXR+ CD3+CD4+, CD8+ T cells: RXR+ CD3+ CD8+, NK cells: RXR+ CD56+, B cells: RXR+ CD20+, Macrophages: RXR+ HLA DR+ CD14+, Plasmacytoid dendritic cells: RXR+ LIN- HLA DR+ CD123+ (pDC), Monocytic dendritic cells: RXR+ LIN- HLA DR+ CD11c+ (mDCs), and myeloid derived suppressor cells (MDSC) Monocytic MDSC (mMDSC) RXR+HLA DR- CD14+CD11b+ and plasmacytic MDSC (pMDSC) RXR+ HLA DR- LIN- CD33+CD11b+. **B, C, and D** 1.5×10^6 peripheral blood mononuclear cells from women without breast cancer ($n=8$) were stimulated by increasing concentrations (0 μM , 5 μM , 10 μM , and 20 μM) of bexarotene for 48 hours. Monocytoid dendritic cells (CD11c+HLA DR+ LIN-) (**B**) and plasmacytoid dendritic cells (CD123+ HLA DR+ LIN-) (**C**) have increased CD40+ expression suggesting increased activation while there was no increase of CD40+ in macrophages (CD14+ HLADR+) (**D**). * $p < 0.05$.

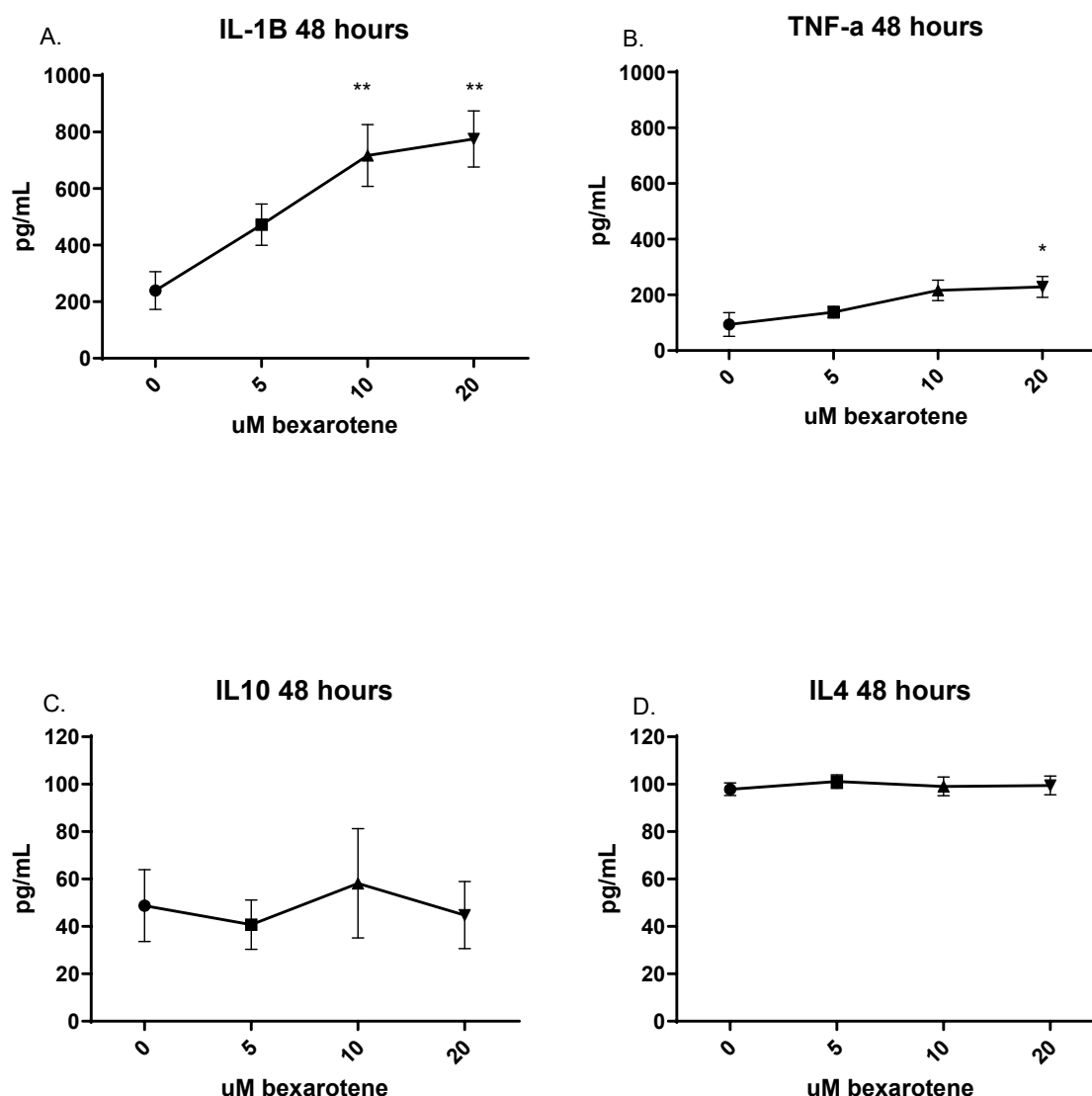


Figure 4. Bexarotene induces predominantly type I, not type II, dendritic cells. 1.5×10^6 peripheral blood mononuclear cells from women without breast cancer ($n=8$) were stimulated by increasing concentrations of bexarotene for 48 hours. PBMC released increased type I IL-1 β (A) and TNF- α (B) but no increase in IL-4 (C) or IL-10 (D) by multiplex cytokine assay. * $p < 0.05$ ** $p < 0.01$.

Increasing dosing of bexarotene induces direct activation of human mDCs

As we demonstrated that bexarotene activated type I mDCs when treating human PBMC, we wanted to show that this activation was direct. CD14 $^{+}$ monocytes were matured into both immature (maintained on human GM-CSF and IL4) or mature (further matured with 48 hours of with IL-1 β , TNF- α , IFN- α , IFN- γ , Poly I:C, IL-2, and IL-7) mDC. The percentage of mDC expressing CD80 and CD86 did not change with doses of bexarotene (data not shown) but bexarotene did increase

CD40 $^{+}$ expression on immature and mature mDC. For immature mDC, 10 μ M bexarotene had $3.2 \pm 0.05\%$ ($p=0.04$) and 20 μ M bexarotene had $3.3 \pm 0.2\%$ ($p=0.05$) as compared to 0 μ M bexarotene which had $1.8 \pm 0.3\%$ CD40 $^{+}$ HLA DR $^{+}$ CD11b $^{+}$ cells/CD40 $^{+}$ HLA DR $^{+}$ cells (Figure 5A). Mature mDC had a larger proportion of mDC expressing CD40 $^{+}$ but this also is increased with bexarotene. 20 μ M bexarotene had $35.7 \pm 0.2\%$ ($p=0.05$) as compared to 0 μ M bexarotene which had $14.3 \pm 2.8\%$ CD40 $^{+}$ HLA DR $^{+}$ CD11b $^{+}$ cells/CD40 $^{+}$ HLA DR $^{+}$ cells (Figure 5B).

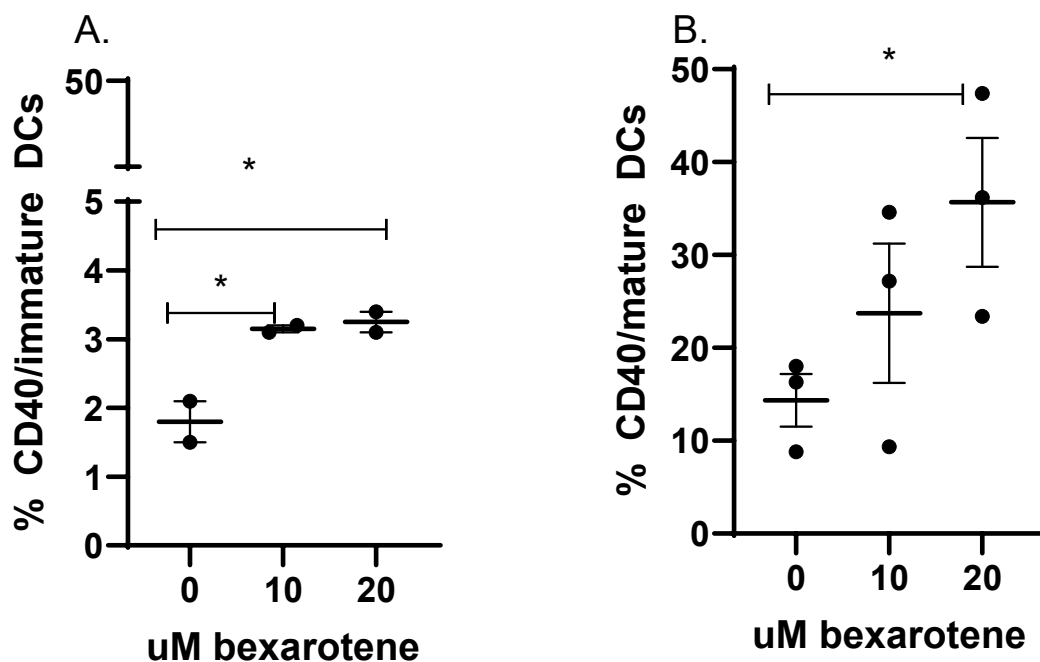


Figure 5. Bexarotene directly activates type I dendritic cells in human PBMC. CD14⁺ monocytes were isolated from human cryopreserved PBMC (n=3) from the Earle A. Chiles immune monitoring laboratory repository and matured with cytokines *in vitro*. Immature dendritic cells were maintained for 48 hours with human GM-CSF and IL4. Mature dendritic cells were matured for 48 hours with IL-1 β , TNF- α , IFN- α , IFN- γ , Poly I:C, IL-2, and IL-7. Immature mDCs (A) and mature mDCs (B) were evaluated for CD40. mDCs are the LIN⁻ CD14⁺ HLADR⁺ CD11c⁺ population. * p<0.05.

Discussion

Bexarotene has previously been shown to prevent tumor development and increase CD8⁺ T cells in TgMMTV-neu mice [19,21]. We further have demonstrated that bexarotene enhanced inhibition of tumor growth along with chemotherapy in both TgMMTV-neu and C3(1)Tag mice with established tumors (Figure 1 and Supplemental Figure 1). While bexarotene did at least double CD8⁺ T cells and decrease CD4⁺ T cells by approximately 3 fold in the tumor in both mouse models it was not higher when combined with chemotherapy as compared to bexarotene or chemotherapy alone except with doxorubicin (Figure 2 and Supplemental Figure 2). Based on our previous data on the immune environment of TgMMTV-neu and C3(1)Tag mice, we have shown that the spontaneous tumors have predominantly Th2 CD4⁺ T cell infiltrate therefore reduction of CD4⁺ T cells suggests a more antitumor proinflammatory immune response [25]. We therefore wanted to determine what direct role bexarotene was playing the immune system that could contribute to its anti-tumor immune role. In human PBMC, the RXR α receptor had the highest expression in APCs (pDCs, mDCs, and macrophages, Figure 3A). Increasing doses of bexarotene induced activated type I mDCs and pDCs but not

macrophages that released increased IL-1 β and TNF α (Figures 3B–3D and Figure 4). We demonstrated that bexarotene is directly activating human mDCs (Figure 5). Chemotherapies have been shown to impact the tumor immune environment but their cytotoxicity still induces systemic immune suppression. Our data suggests that bexarotene can enhance chemotherapy efficacy and increase CD8⁺ T cells in the tumor and based on the human data, may be through increasing type I dendritic cell activation.

Chemotherapy induces apoptosis and decreases cell proliferation of tumor cells. However chemotherapy has also been shown to have dynamic roles in immune responses in the tumor immune environment. Cyclophosphamide has been shown to reduce regulatory T cells and multiple inflammatory mediators including GM-CSF, IL-1 β , IFN- γ , and TNF α [26–28]. However, cyclophosphamide also recruits MDSC to the tumor immune environment inducing immune suppression [29]. Similarly, paclitaxel has been shown to induce TNF α and activate TLR-4 in the tumor immune environment which are both associated with immune activation [29]. Paclitaxel also induces chronic inflammation driving angiogenesis and increasing immunosuppressive cytokines IL6 and IL8 [30]. Doxorubicin induces TLR4 activation and the production of

inflammatory cytokines and reactive oxygenation species [31–33]. Cisplatin can induce reactive oxygenation species and induce inflammation [34]. For all of these chemotherapies, the balance of the pro and anti-inflammatory effects impacts chemotherapy efficacy and chemoresistance [35]. A therapy that can enhance the anti-tumor efficacy of chemotherapy could improve the efficacy of chemotherapy. The impact of CD8+ T cell infiltrate on anti-tumor immune function appears to be chemotherapy and mouse model specific. For example, in TgMMTV-neu mice, combining bexarotene with doxorubicin had decreased CD8+ T cell infiltrate but also improved tumor inhibition (**Figure 1A** and **2A**). In C3(1)Tag mice, bexarotene and doxorubicin had increased CD8+ T cell infiltrate compared to doxorubicin ($p=0.004$) (**Supplemental Figure 2A**) but did not significantly improve tumor inhibition because was so effective with doxorubicin alone. (**Supplemental Figure 1A**). We demonstrate that bexarotene enhances anti-tumor efficacy when combined with chemotherapy (**Figure 1** and **Supplementary Figure 1**) in two independent mouse mammary tumor models and both chemotherapy and bexarotene increase CD8+ T cells while decreasing CD4+ T cells in the tumor but the direct impact of bexarotene on the increased CD8+ T cells remains to be shown.

Bexarotene has previously been associated with an anti-tumor immune response. Bexarotene decreases malignant regulatory CD4+ T cells in cutaneous T cell lymphoma and increases CD8+ T cells in TgMMTV-neu tumors that develop during treatment with bexarotene [21,36]. The presence of T cells, particularly cytotoxic T cells, have been associated with improved prognosis across cancer types, including breast cancer [3,37]. However, the presence of cytotoxic T cells in the stroma or infiltrating the tumor are lower in untreated breast cancer than other more immune active cancer and this is believed to be because of immune suppressive populations in the tumor immune environment particularly the innate immune cells [1,38]. In human PBMC, only ~5% of peripheral CD4+ T cells and ~3% of peripheral CD3+ T cells expressed RXR α and there was no expression in NK cells or B cells. The highest expression of RXR α was in APCs (macrophages, pDC, and mDC) which were ~30% of the cells expressing RXR α (**Figure 3A**). Professional APCs are important to present tumor antigens to T cells in a cytokine milieu that determines whether those anti-tumor T cells to be active [39]. Increased type 1 conventional dendritic cells (mDCs) have been associated with improved prognosis across cancer types [40,41]. In breast cancer, the presence of mDC has been associated with improved survival [40]. Increasing doses of bexarotene increased activation of type I mDCs and pDCs but not macrophages (**Figures 3B–3D** and **4**). Bexarotene was able to directly induce type I mDC (**Figure 5**). Bexarotene may be inhibiting tumor growth and increasing type I CD8 T cells by activating type I dendritic cells which can increase tumor-antigen specific CD8+ T cells that can infiltrate the tumor and

decrease CD4+ T cells allowing those CD8+ T cells to be more active. This study did not directly show the impact of dendritic cell activation in tumor inhibition in the mouse models.

This data has demonstrated that bexarotene can directly activate human type I dendritic cells and can increase CD8+ T cells in two transgenic mouse models of breast cancer enhancing tumor inhibition with chemotherapy. One limitation of the study is that dendritic cell activation was not shown to be necessary for the anti-tumor effect in mice or humans therefore further studies are needed. There is a current trial NCT02876640 that treated patients with early breast cancer with 2 weeks of the rexinoid 9cUAB30 (a rexinoid that specifically targets endothelial cells) that will be evaluating the impact of the rexinoid on the activation of systemic dendritic cells but again does not directly evaluate the anti-tumor impact of the dendritic cells [42]. Our data support using bexarotene in combination with chemotherapy to enhance the function of the chemotherapy in breast cancer.

Conclusions

Bexarotene enhances the function of chemotherapy in breast cancer, increasing CD8+ T cells and decreasing CD4+ T cells in TgMMTV-neu and C3(1)Tag transgenic mouse models. In human PBMC, RXR α is expressed highest in APCs, and can activate type I mDC and pDC. This suggests that bexarotene's improved anti-tumor benefit with chemotherapy has an immune component that increases CD8+ T cells in the tumor. Further work is needed but this may be through activation of type I dendritic cells.

List of Abbreviations

pDCs: Plasmacytic Dendritic Cells; mDCs: Monocytic Dendritic Cells; MDSC: Myeloid Derived Suppressor Cells; FFPE: Formalin Fixed Paraffin Embedded; TILs: Tumor Infiltrating Lymphocytes

Declarations

Ethics approval

All mouse work was approved by the University of Washington Mary L. Disis IACUC and all repository clinical samples were collected from the University of Washington Cancer Vaccine Institute Repository approved by the Fred Hutchinson IRB.

Consent for publication

No personal identifying information is included in this manuscript.

Availability of data and materials

All data are available by reasonable request from Sasha Stanton MD PhD.

Competing interests

MLD has grant funding from Precigen, Veanna, Bavarian Nordic, Aston Sci. Founder of Epithany. Inventor on patents held by University of Washington. Royalties received from University of Washington. SES has research funding from Veanna and Ataraxis and drug support for a clinical trial from Canwell, Inc. LC, EG, JS, and MY have no conflicts to declare.

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Author's contributions

Conceptualization: SES and MLD; Data Curation: SES; Formal Analysis: LC and MY; Supervision: SES and MLD; Investigation: SES, JS, and MY; Methodology: SES; Writing original draft: SES; Reviewing and Editing: SES, MLD, and LC.

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