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Post-transcriptional control of PD-L1 expression by 17 β -estradiol via PI3K/Akt signaling pathway in ER α -positive cancer cell lines

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Abstract

Objective—Estrogen is a well-known oncogenic driver in endometrial and breast cancers.

Programmed cell death protein 1 (PD-1) and its ligands PD-L1 and PD-L2 have been shown to mediate immune evasion of the tumor cells. The purpose of the present study was to assess the effects of estrogen on PD-L1 and PD-L2 expression in endometrial and breast cancer cell lines.

Methods—17 β -estradiol (E2) -induced expression of PD-L1 and PD-L2 and possible signaling pathway were investigated in endometrial and breast cancer cells. Co-culture of T cells and cancer cells with E2 stimulation was performed to assess the functions of T cells.

Results—We found that 17 β -estradiol (E2) increased expression of PD-L1, but not PD-L2 protein via activation of phosphoinositide 3-kinase (PI3K)/Akt pathway in Ishikawa and MCF-7 cells. PI3K and Akt inhibitors could block E2's effects. E2 did not increase PD-L1 mRNA transcription, but stabilized PD-L1 mRNA. E2's effects were only observed in estrogen receptor α (ER α) -positive Ishikawa and MCF-7 cells, but not in ER α -negative MDA-MB-231 cells. Co-culture of Ishikawa or MCF-7 cells with T cells inhibited expression of interferon- γ and interleukin-2 and increased Bim expression in the presence of E2.

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Conclusion—This study provides the first evidence that estrogen up-regulates PD-L1 protein expression in ER α -positive endometrial and breast cancer cells to suppress immune functions of T cells in the tumor microenvironment, demonstrating a new mechanism of how estrogen drives cancer progression.

Keywords

Estrogen; PD-L1; PI3K; Akt; Endometrial cancer; Breast cancer

Introduction

Endometrial cancer (EC) and breast cancer (BC) are two common malignancies in women worldwide¹. Type I EC includes endometrial adenocarcinoma that represents 80% to 90% EC arising from atypical endometrial hyperplasia with unopposed estrogen exposure^{2, 3}. Likewise, increased lifetime exposure to estrogen as inferred by early menarche, late menopause, or obesity is associated with an increased BC risk^{4, 5}. The majority of EC and BC are estrogen-dependent adenocarcinomas with estrogen receptor α (ER α) expression. Estrogen-stimulated cellular proliferation remains the conceptual underpinning of ER α -dependent mechanism in EC and BC development and progression⁶.

Recently, the B7-CD28 family of immune checkpoint proteins has been demonstrated to play key roles in regulating T-cell activation and immunological tolerance⁷. T cells, natural killer cells, monocytes, and B cells have been shown to express programmed cell death protein 1 (PD-1), a member of the B7-CD28 family^{8, 9}. The ligands for PD-1 (PD-Ls) are PD-L1 (also known as B7-H1) and PD-L2 (also named B7-DC), both of which can be found not only on immune cells, but also in cancer cells including lung cancer, ovarian cancer, colon cancer, and melanoma¹⁰⁻¹². Tumor-associated PD-L1 can be induced by various factors, including interferon (IFN) family, tumor necrosis factor α , vascular endothelial growth factor, and cytokines such as interleukin-4 (IL-4) and IL-10^{10, 13-15}. In the tumor microenvironment, PD-Ls act through PD-1 to inhibit T-cell proliferation, reduce T-cell activation, and induce T-cell apoptosis^{9, 16, 17}. Substantial preclinical and clinical evidences have proved that PD-1/PD-Ls play a major role in immune suppression within the tumor microenvironment and anti-PD-1/PD-L1 antibodies are effective in the treatment of multiple cancers^{10, 18-21}. Therefore, the United States Food and Drug Administration has approved two anti-PD-1 monoclonal antibodies (nivolumab and pembrolizumab) for treatment of unresectable or metastatic melanoma, non-small-cell lung carcinoma (NSCLC), and metastatic renal cell carcinoma, based on clinical efficacy and safety data²⁰⁻²³. Aside from anti-PD-1 antibodies, anti-PD-L1 atezolizumab has been shown to be efficacious in bladder cancer and NSCLC²⁴⁻²⁶ and has recently been approved for treatment of locally advanced or metastatic urothelial carcinoma.

Previously we have studied PD-1/PD-Ls in human lung cancer²⁷, human cervical intra-epithelial neoplasia²⁸, and mouse prostate cancer²⁹. Particularly, we have found that 61.3% of ECs were positive for PD-1 expression and PD-L1/2 expression was increased in poorly differentiated ECs³⁰. Therefore, we became interested in investigating the factors that could regulate the expression of PD-Ls in cancer cells. Since estrogen is a well-known oncogenic

driver in EC and BC and it is not known whether 17 β -estradiol (E2) can regulate PD-Ls expression in cancer cells, we conducted this study with the aim to assess the effects of E2 on PD-Ls expression in EC and BC cells.

Materials and Methods

Cell culture

Human endometrial cancer cell line Ishikawa (ER α -positive), human breast cancer cell lines MCF-7 (ER α -positive) and MDA-MB-231 (ER α -negative), and Jurkat cells (immortalized from acute T cell leukemia and often used as T lymphocytes) were purchased from the American Type Culture Collection (Manassas, VA, USA) and were free of mycoplasma contamination. Human primary T cells were isolated from donated blood and obtained from State/National Key Laboratory of Biotherapy, Sichuan University. The cells were cultured in Dulbecco's Modified Eagles Medium (DMEM, Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Fisher Scientific) and 100 U/mL penicillin/streptomycin in a humidified incubator with 5% CO₂ at 37°C. Jurkat and primary T cells were cultured in RPMI-1640 medium (Fisher Scientific) supplemented with 10% FBS and 100 U/mL penicillin/streptomycin in a humidified incubator with 5% CO₂ at 37°C.

Reagents

E2, cycloheximide (CHX) and actinomycin D (ActD) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Pan-Akt inhibitor AZD5363 was obtained from Selleck Chemicals, Inc. (Houston, TX, USA). Phosphoinositide 3-kinase (PI3K) inhibitor LY294002 was obtained from Cell Signaling Technology (Danvers, MA, USA). The primary antibodies used were: rabbit anti-PD-L1 monoclonal antibody (#ab205921, Abcam, Cambridge, MA, USA), rabbit anti-PD-L2 polyclonal antibody (#SAB3500395, Sigma-Aldrich), rabbit anti-ER α polyclonal antibody (#MC-20, Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-phospho-Akt^{Ser473} polyclonal antibody (#9271) and rabbit anti-Akt polyclonal antibody (#9272)(Cell Signaling Technology), mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (#MAB374, Millipore, Billerica, MA, USA), anti-human PD-L1 neutralizing antibody (clone MIH1, eBiosciences, San Diego, CA, USA), and isotype mouse IgG (R&D Systems, Minneapolis, MN, USA).

Real-time quantitative RT-PCR

After the indicated time of treatment, cells were harvested for total RNA extraction using NucleoSpin RNA kit (Macherey-Nagel, Bethlehem, PA, USA) according to the manufacturer's instructions. cDNA was synthesized from total RNA using PrimeScript™ reverse transcription kit (Takara, Mountain View, CA, USA). Human PD-L1, PD-L2, and GAPDH primers were obtained from Eurofins MWG Operon (Huntsville, AL, USA). The sequences are as follows: PD-L1, Forward: 5'-CTCAGGGTGACAGAGAGAAG-3', Reverse: 5'-GACACCAACCACCAGGGTTT-3'; PD-L2, Forward: 5'-TGGCATTGCTGACGCATTT-3', Reverse: 5'-TGCAGCCAGGTCTAATTGTTTT-3'; GAPDH, Forward: 5'-CCACATCGCTCAGACACCAT-3', Reverse: 5'-TAAAAGCAGCCCTGGTGACC-3'. Real-time quantitative RT-PCR (qRT-PCR) was performed in triplicates with an iQ5 iCycler and iQ SYBR Green Supermix (Bio-Rad

Laboratories, Hercules, CA, USA) following the manufacturer's protocols. Results were normalized to GAPDH levels using the formula $\text{cycle threshold (Ct)} = \text{Ct of target gene} - \text{Ct of GAPDH}$. The mRNA level of the control group (vehicle treatment) was used as the baseline; therefore, Ct was calculated using the formula $\text{Ct} = \text{Ct of target gene} - \text{Ct of the baseline}$. The fold change of mRNA level was calculated as $\text{fold} = 2^{-\text{Ct}}$.

Western blot

After the indicated time of treatment, proteins were extracted from the cells in RIPA lysis buffer (50 mM sodium fluoride, 0.5% Igepal CA-630 [NP-40], 10 mM sodium phosphate, 150 mM sodium chloride, 25 mM Tris pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 2 mM ethylenediaminetetraacetic acid [EDTA], 1.2 mM sodium vanadate) supplemented with protease inhibitor cocktail (Sigma-Aldrich). Equal amount of proteins was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% nonfat dry milk in TBS-T buffer (25 mM Tris-HCl, 125 mM NaCl, 0.1% Tween-20) for 1 hour and probed with the indicated primary antibodies overnight and then IRDye 800CW- or IRDye 680RD-conjugated secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA) for 1 hour. The results were visualized using an Odyssey Infrared Imager (LICOR Biosciences). For loading control, the membranes were stripped and probed for unphosphorylated proteins and/or GAPDH. Quantification of the Western blot signals was performed using the image analysis software of the Odyssey Infrared Imager system. The integrated density values of signals of targeted genes were normalized by those of GAPDH. The ratio indicates the relative level of target protein.

Functional analysis of T cells after co-cultured with Ishikawa or MCF-7 cells

Jurkat cells and human primary T cells were used for functional analysis. Previous studies have found that Jurkat cells express low basal levels of PD-1 mRNA and protein, which is responsive to anti-PD-L1 antibody^{31, 32}. We also confirmed that Jurkat cells express PD-1 mRNA and protein before using this cell line (data not shown). 1×10^6 of T cells (Jurkat or primary T cells) and 1×10^6 of cancer cells (Ishikawa or MCF-7 cells) were co-cultured in 6-cm dishes. In addition, anti-human PD-L1 (1 $\mu\text{g/mL}$) or isotype mouse IgG (1 $\mu\text{g/mL}$) were added to cancer cells. After incubation for 2 hours, the antibody-treated cancer cells were added to dishes containing T cells. After 16 hours serum starvation, cells were treated with 10 nM E2 for 8 hours. Then, the cancer cells grew adherently to the dishes, while the suspending T cells were harvested for RNA isolation and analyzed for expression of interferon- γ (IFN- γ), IL-2 and BCL-2-interacting mediator of cell death (Bim) mRNA using qRT-PCR as described above. The PCR primer sequences are as follows: IFN- γ , Forward 5'-CTAATTATTCGGTAACTGACTTGA-3', Reverse 5'-ACAGTTCAGCCATCACTTGGA-3'; IL-2, Forward 5'-TGCCACAATGTACAGGATGC-3', Reverse 5'-GCCTTCTTGGGCATGTAAAA-3'; Bim, Forward 5'-GCCGCCACTACCACCACTT-3', Reverse 5'-AACCGAATACCGCGATGATG-3'.

Statistical analysis

Statistical analysis was performed using SPSS (v17.0, SPSS Inc., Chicago, IL, USA). Data are presented as mean $\pm$ standard error (SE). Quantitative data were analyzed using Student's *t*-test (two-tailed). Statistical significance was reached with a *P* value less than 0.05.

Results

E2 does not regulate PD-L1 and PD-L2 mRNA expression

To assess E2's effects on PD-L1 and PD-L2 expression, we tested two cancer cell lines (Ishikawa and MCF-7) with ER α protein expression and one cancer cell line (MDA-MB-231) without ER α protein expression as negative control. We first confirmed that Ishikawa and MCF-7 cell lines were ER α -positive and MDA-MB-231 cell line was ER α -negative (Fig. 1A). We found that E2 at dosages from 1 to 10 nM did not induce significant changes in PD-L1 and PD-L2 mRNA levels (*P*>0.05, Fig. 1B). E2 at a dosage of 10 nM did not induce significant changes in PD-L1 and PD-L2 mRNA levels over a time-course of 2 to 24 hours (*P*>0.05, Fig. 1C).

E2 increases PD-L1 protein expression in ER α -positive cells via activation of PI3K/Akt pathway

We found that E2 at dosages of 1 to 10 nM significantly increased PD-L1, but not PD-L2, protein expression in Ishikawa (Fig. 2A) and MCF-7 (Fig. 2B) cell lines, but not in MDA-MB-231 cell line (Fig. 2C). E2 at a dosage of 10 nM increased PD-L1, but not PD-L2, protein expression in a time-dependent manner in Ishikawa (Fig. 3A) and MCF-7 (Fig. 3B) cell lines, but not in MDA-MB-231 cell line (Fig. 3C). Akt activation, based on phosphorylated Akt (p-Akt) levels, appeared to coincide with or precede the increased PD-L1 protein levels in Ishikawa (Fig. 3A) and MCF-7 (Fig. 3B) cell lines, but not in MDA-MB-231 cell line (Fig. 3C). Of note, total Akt (t-Akt) levels were also increased by E2 treatment in Ishikawa cell line (Fig. 3A).

To determine if E2-activated PI3K/Akt signaling pathway regulated PD-L1 protein expression, we treated Ishikawa and MCF-7 cells with a pharmacologic inhibitor of PI3K (LY294002) and a pan-Akt inhibitor AZD5363. We found that LY294002 completely blocked Akt activation and decreased E2-induced PD-L1 protein expression in Ishikawa and MCF-7 cells (Fig. 4A, B). Consistently, AZD5363 maintained p-Akt levels but decreased E2-induced PD-L1 protein expression in Ishikawa and MCF-7 cells (Fig. 4C, D).

E2 increases PD-L1 mRNA stability but not protein stability through activation of Akt

To explore the mechanisms of how E2 increased PD-L1 protein expression, we checked PD-L1 mRNA levels in Ishikawa and MCF-7 cells after blocking transcription by ActD treatment. We found that PD-L1 mRNA levels quickly decreased in the presence of ActD, however, with addition of 10 nM E2, the decrease of PD-L1 mRNA levels were significantly slowed down. When 2 μ M AZD5363 was added to inhibit Akt function, E2 was unable to stabilize PD-L1 mRNA levels (Fig. 5A). In contrast, when protein translation was blocked by CHX treatment, E2 did not change the rate of decrease at PD-L1 protein levels in Ishikawa and MCF-7 cells (Fig. 5B).

E2 inhibits T-cell function in co-culture of Ishikawa or MCF-7 and T cells

To determine if E2-induced PD-L1 protein expression was immunologically relevant, Jurkat or primary T cells were co-cultured with cancer cells (Fig. 6A, B, F, G). We found that E2 significantly increased IL-2 mRNA levels in Jurkat cells (Fig. 6C), but not in primary T cells (Fig. 6H), in the absence of co-culturing with any cancer cells. However, in co-culture of Ishikawa or MCF-7 and Jurkat or primary T cells, E2 significantly decreased mRNA levels of IFN- γ and IL-2 in Jurkat and primary T cells (Fig. 6D, E, I, J). Anti-PD-L1 antibody, but not IgG, reversed E2-induced down-regulation of IFN- γ and IL-2 in Jurkat and primary T cells (Fig. 6D, E, I, J). Of note, E2 did not affect IFN- γ or Bim mRNA expression in the absence of co-culture with cancer cells (Fig. 6C, H). In contrast, E2 slightly increased Bim mRNA levels in Jurkat cells co-cultured with Ishikawa or MCF-7 cells, and the increase reached statistical significance in only MCF-7 cells (Fig. 6D, E). In addition, in co-culture of Ishikawa or MCF-7 and primary T cells, E2 significantly increased Bim mRNA level in primary T cells. Yet, E2-induced Bim up-regulation was abolished by anti-PD-L1 antibody, but not by IgG (Fig. 6E, I, J).

Discussion

Cancer development and progression require evasion of immune attack through up-regulation of immune inhibitory factors in cancer cells³³. One of these inhibitory factors is PD-L1, which binds to PD-1 expressed on T cells, B cells, natural killer cells, and dendritic cells to suppress anti-cancer immunity. On the other hand, it is not a bad thing to have high levels of PD-L1 expressed in cancer cells, since there are a number of studies indicating that PD-L1 expression in cancer may be a good prognosis factor for cancer patients in certain cases. For example, PD-L1 mRNA expression is identified in nearly 60% of breast tumors and it is associated with increased number of tumor infiltrated lymphocytes and improved recurrence-free survival³⁴. PD-L1 expression is often associated with high number of tumor infiltrated lymphocytes, thus providing a good target for anti-PD-1/PD-L1 blockade therapy³⁵. In the present study, we found that E2 remarkably increased PD-L1 protein expression in dose- and time-dependent manners, suggesting that E2 can regulate anti-cancer immunity. Indeed, we found that E2 inhibited T-cell functions in co-culture of Jurkat or primary T cells with cancer cells. It is well known that E2 stimulates cancer cell proliferation to drive cancer development and progression⁶. The present study adds a new role of E2 in suppressing anti-cancer immunity through increasing PD-L1 protein expression in endometrial and breast cancer cells.

Interestingly, PD-L2 expression was not affected by E2, suggesting that the regulatory mechanisms for PD-L1 and PD-L2 expression are different. E2 acts through ER α , which was why E2-induced PD-L1 protein expression was found in ER α -positive Ishikawa and MCF-7 cells, but not in ER α -negative MDA-MB-231 cells. In contrast to induction of PD-L1 protein expression, E2 did not affect PD-L1 mRNA expression (Fig. 1B, C). At 10 nM dosage, E2 only slightly increased PD-L1 mRNA levels (Fig. 1B), which was not significant statistically. Since E2 did not affect PD-L1 mRNA transcription, we focused on non-genomic actions of E2. Previous studies have demonstrated that E2 rapidly activates PI3K/Akt signaling pathway in Ishikawa and MCF-7 cells^{36, 37}, which is dependent on

membrane ER α binding to p85 α regulatory subunit of PI3K^{38, 39}. It has been well established that E2 can activate PI3K/Akt signaling pathway^{40, 41}, which is consistent with our findings in the present study. We showed that activation of PI3K/Akt is essential for E2-induced PD-L1 protein expression, because PI3K and Akt inhibitor can completely block E2's effects. Of note, we observed that Akt was maintained in a hyperphosphorylated but catalytically inactive form in Ishikawa and MCF-7 cells after AZD5363 treatment. This phenomenon was also reported by other investigators^{42, 43}. One possible mechanism is that Akt inhibitor sensitizes the Akt pleckstrin homology (PH) domain binding to phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which facilitates membrane localization of Akt and induces conformational change of Akt, making it more susceptible to kinase phosphorylation or less susceptible to phosphatase dephosphorylation⁴⁴. A previous study has shown that human immunodeficiency virus can activate PI3K/Akt to up-regulate PD-L1 expression⁴⁵. Further, it has been shown that PI3K/Akt regulates PD-L1 expression at post-transcriptional levels⁴⁶. We found that E2 increased PD-L1 mRNA stability, but not protein stability. This is consistent with a previous report that PI3K/Akt can inhibit mRNA degradation⁴⁷.

T cells express IFN- γ to attack target cells and IL-2 to stimulate proliferation and survival of T cells^{9, 14}. We found that E2 treatment decreased expression of IFN- γ and IL-2 in Jurkat or primary T cells co-cultured with cancer cells, suggesting that E2-induced PD-L1 protein in cancer cells inhibits T-cell functions. On the other hand, E2 increased expression of Bim, an initiator of T-cell apoptosis⁴⁸, in Jurkat or primary T cells co-cultured with cancer cells, indicating that E2-induced PD-L1 protein may lead to T-cell apoptosis, thus contributing to immunosuppression. It has been demonstrated that Bim is a downstream molecule of PD-1 signaling pathway in T cells^{49, 50}. Thus, it is worth pointing out that E2 increases Bim expression through induction of PD-L1, as treatment with anti-PD-L1 antibody can abolish E2-induced Bim up-regulation in Jurkat and primary T cells (Fig. 6E, I, J).

In conclusion, the present study provides the first evidence that E2 activates PI3K/Akt pathway to increase PD-L1 protein expression in ER α -positive endometrial and breast cancer cells, which is through a post-transcriptional mechanism to stabilize PD-L1 mRNA. Our results demonstrate a new role of estrogen in regulation of anti-cancer immunity, implying PD-L1 as a potential therapeutic target and/or an outcome parameter in anti-estrogen treatment of endometrial and breast cancers.

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Figure 1.

E2's effects on PD-L1 and PD-L2 mRNA expression in cancer cells. (A) Western blot analysis of ERα expression; GAPDH serves a loading control. ERα levels in Ishikawa and MCF-7 cell lines are presented as mean ± standard error of 3 independent measurements based on Western blot analysis. ERα expression is not detectable in MDA-MB-231 cell line. (B) Dose-dependent effects of E2 on PD-Ls mRNA levels analyzed by qRT-PCR and quantitated as a fold change against the control by using the 2^{-CT} method. (C) Time-dependent effects of E2 on PD-Ls mRNA levels analyzed by qRT-PCR and quantitated as a fold change against the control by using the 2^{-CT} method. Data represent mean ± SE of 3 independent experiments.

Figure 2.

Dose-dependent effects of E2 on PD-L1 and PD-L2 protein expression in cancer cells assessed by Western blot analysis. The integrated density values of signals of targeted genes were normalized by those of GAPDH based on Western blot analysis. Relative protein levels were calculated by the ratio of PD-L1/GAPDH or PD-L2/GAPDH. Data are presented as mean $\pm$ standard error of 3 independent experiments. (A) Ishikawa cell. (B) MCF-7 cell. (C) MDA-MB-231 cell.

Figure 3.

Time-dependent effects of E2 on PD-L1 and PD-L2 protein expression and Akt activation in cancer cells assessed by Western blot analysis. The integrated density values of signals of targeted genes were normalized by those of GAPDH based on Western blot analysis.

Relative protein levels were calculated by the ratio of p-Akt/GAPDH, t-Akt/GAPDH, PD-L1/GAPDH or PD-L2/GAPDH. Data are presented as mean $\pm$ standard error of 3 independent experiments. (A) Ishikawa cell. (B) MCF-7 cell. (C) MDA-MB-231 cell.

*P<0.05, compared to the control group.

Figure 4. Effects of PI3K inhibitor LY294002 (A, B) and pan-Akt inhibitor AZD5363 (C, D) on E2-induced PD-L1 protein expression. Cancer cells were treated with 10 nM E2, with or without 10 μ M LY294002 and 2 μ M AZD5363; PD-L1 protein levels were assessed by Western blot analysis. The integrated density values of signals of targeted genes were normalized by those of GAPDH based on Western blot analysis. Relative protein levels in were calculated by the ratio of p-Akt/GAPDH, t-Akt/GAPDH or PD-L1/GAPDH. Data are presented as mean $\pm$ standard error of 3 independent experiments.

Figure 5.

E2's effects on PD-L1 mRNA and protein stability. (A) Cancer cells were exposed to 5 $\mu\text{g/mL}$ ActD, with or without 10 nM E2 and 2 μM AZD5363 for the indicated time points; PD-L1 mRNA levels were assessed by qRT-PCR; data represent mean $\pm$ SE, * $P < 0.05$, ** $P < 0.01$. (B) Cancer cells were exposed to 50 $\mu\text{g/mL}$ CHX, with or without 10 nM E2 for the indicated time points; PD-L1 protein levels were assessed by Western blot analysis. The integrated density values of signals of targeted genes were normalized by those of GAPDH

based on Western blot analysis. Relative protein levels were calculated by the ratio of PD-L1/GAPDH. Data are presented as mean $\pm$ standard error of 3 independent experiments.

Figure 6.

E2's effects on T-cell functions. Photomicrographs of Jurkat cells co-cultured with Ishikawa (A) or MCF-7 (B) cells. The small round cells in suspension are Jurkat cells (white arrows). The large flat adherent cells are cancer cells (black arrows). (C) Jurkat cells were treated with 10 nM E2 for 8 hours; mRNA levels were determined using qRT-PCR. (D) After co-cultured with Ishikawa cells, with or without IgG or anti-PD-L1 antibodies (Abs) treatment, followed with 10 nM E2 treatment for 8 hours, total RNA of Jurkat cells was isolated for qRT-PCR. (E) After co-cultured with MCF-7 cells, with or without IgG or anti-PD-L1 antibodies (Abs) treatment, followed with 10 nM E2 treatment for 8 hours, total RNA of Jurkat cells was isolated for qRT-PCR. Photomicrographs of primary T cells co-cultured with Ishikawa (F) or MCF-7 (G) cells. The small round cells in suspension are primary T cells (white arrows). The large flat adherent cells are cancer cells (black arrows). (H) Primary T cells were treated with 10 nM E2 for 8 hours; mRNA levels were determined using qRT-PCR. (I) After co-cultured with Ishikawa cells, with or without IgG or anti-PD-L1 antibodies (Abs) treatment, followed with 10 nM E2 treatment for 8 hours, total RNA of primary T cells was isolated for qRT-PCR. (J) After co-cultured with MCF-7 cells, with or without IgG or anti-PD-L1 antibodies (Abs) treatment, followed with 10 nM E2 treatment

for 8 hours, total RNA of primary T cells was isolated for qRT-PCR. Data represent mean $\pm$ SE of 3 independent experiments.

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