Rapid, no-wash measurement of immune checkpoint molecules expression induced by interaction with peripheral blood mononuclear cells in breast and cervical cancer cell models

#4671

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1 Introduction

Programmed cell death ligand 1 (PD-L1) expression increases with tumor severity in basal-like breast cancer and is enhanced in cervical cancers. Basal-breast tumors can adapt to lymphocytic infiltration by responding to heightened concentrations of interferon gamma (IFN-γ) secreted by Type 1 helper T cells with upregulation of PD-L1 protein allowing the tumors to evade immune targeting and reduce the immune response. We show here that human peripheral blood mononuclear cells (PBMCs) secrete IFN-γ and other cytokines in response to activation with anti-CD3/anti-CD28 coated Dynabeads® (DB). We then examine the modulation in expression of immune checkpoint molecules and secretion of cytokines in HCC38 (basal breast cancer-derived) and HeLa (cervical cancer) cells in response to co-culturing with DB-stimulated PBMCs, treatment with conditioned media collected from activated PBMCs, and direct treatment with recombinant IFN-γ. As expected, IFN-γ treatment induced upregulation or PD-L1 protein expression, an effect largely independent of cellular proliferation and dependent on signaling through the JAK/STAT pathway. The effects of conditioned media and direct co-culturing with PBMCs resulted in more complex effects on PD-L1 expression as well as on secretion of a variety of cytokines measured with AlphaLISA® no-wash assay technology. To determine if tumor cytoarchitecture influences responsiveness to the PBMCs, biomarker expression was also measured from cultures grown in 3D spheroid cultures.

2 Materials and Methods

Cell culture and treatment: HCC38 (ATCC® CRL-2314™) and HeLa (ATCC® CCL-2™) cells were grown in culture media consisting of RPMI-1620 (ATCC® 30-2001™) with 10% FBS (ThermoFisher #26140079) and seeded into 96-well ViewPlates™ (#6005182) or CellCarrier™ Spheroid ULA microplates (#6055330) for experiments. A single vial of human PBMCs (ATCC® PCS-800-011™) were thawed, rinsed, and re-suspended in media for each experiment. Half the cells were activated by treatment with Dynabead Human T-Activator CD3/CD28 (ThermoFisher #11131D) at a standard ratio of one bead to one cell. In some conditions, cultures were treated with recombinant human IFN-γ (BioLegend #570206). For assessment of intracelluar signaling (Section 3, graphs C-D), HCC38 cells were treated with a titration of JAK pathway-specific inhibitors (Ruxolitinib and Tofacitinib; Selleckchem #S1378 and #S2789 respectively) that were added just prior to IFN-γ addition.

For 2D Co-culture experiments, HCC38 and HeLa cells were seeded at 25,000 cells (in 50 µL) per well on day zero. The same day, PBMCs were prepared at a concentration of 500K cells per mL (25K/ 50 µL) and grown for 2 days in T-25 flasks with or without Dynabeads (1 bead: 1 cell ratio). After 2 days, PBMCs were removed from flasks and either added directly to the culture plate containing HCC38 or HeLa cells cells or were spun down to collect supernatant for conditioned media (CM). PBMCs or CM were added at 50 µL/well to the plate containing adherent cells. After 2 days of co-culture (or treatment with CM or IFN-γ), culture plates were spun down briefly (~4 minutes at ~500 RPM), 62 µL supernatants were collected (some media left in order to leave PBMCs), and cells were lysed by adding 4X AlphaLISA Lysis buffer (#AL001F) for ~20 minutes. Supernatants and lysate samples (>55 µL lysate per well) were transferred directly to polypropylene StorPlates (#6008290) and kept up to 2 weeks at -20ºC before assays were run.

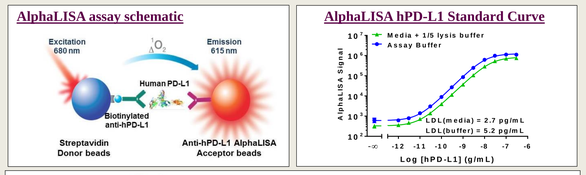
AlphaLISA biomarker quantification assays: Sample plates were thawed and 5 µL of lysate or supernatant samples were added to separate 384-well gray AlphaPlates™ (#6005350) where AlphaLISA assays were performed according to each assay manual. **AlphaLISA** is a **No-Wash, Mix and Read Assay Technology** whereby most assays were completed in **less than 3 hours.** For assays measuring cytokines in supernatant, the following AlphaLISA kits were used: IFN-γ (#AL327), IL-6 (#AL3025), TNF-α (#AL325), IL-1β (#AL220), IL-17A (#AL219), and IL-6 (#AL3025). For protein detection from lysate, we measured: PD-L1 (#AL355), CTLA-4 (#AL3050), TIM-3 (#AL3052), Galectin-9 (GAL-9, #AL3051).

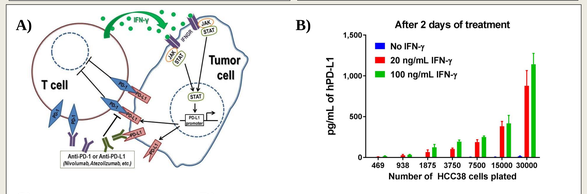
AlphaLISA assays were measured on a EnVision® multilabel plate reader using standard Alpha Settings. Titrations of recombinant standards of each biomarker measured were run alongside samples being tested in the same sample matrix (culture media or media+lysis buffer) and raw data were interpolated to the standard curve to determine pg/mL of protein in each sample. For detection of phosphorylated STAT1, HCC38 cultures were lysed with Alpha *SureFire® Ultra* lysis buffer (#ALSU-LB-10mL) for 10 minutes and samples assayed using AlphaLISA SureFire *Ultra* kits (#ALSU-PST1-A500 & #ALSU-PST1-B500).

ATPlite™ 1step and ATPlite™ 1step 3D assays: Cellular proliferation and viability were assessed by examining ATP content in cultures using ATPlite 1step and ATPlite 1step 3D assays following kit protocols. For 3D cultures, the assay was run in the clear Spheroid plate and 50 uL samples were transferred to a white ½ AreaPlate-96 (#6005560) for measurement. Luminescence was read on the EnSight™ multimode plate reader.

3D staining and imaging: For staining 3D cultures, cells were labeled with the addition of Hoechst 33342, Calcein-AM, and Propidium Iodide (ThermoFisher, #H3570, C3100MP, and #P3566 respectively) dyes to culture media and incubated for less than 1 hour before imaging on the Operetta® High Content Imager using the 10X long WD objective and appropriate fluorescence optics. For assessment of cell number in monolayer wells (Section 5, graph B), cultures were labeled with Hoechst, imaged with the EnSight plate reader and cells automatically counted using the count nuclei function.

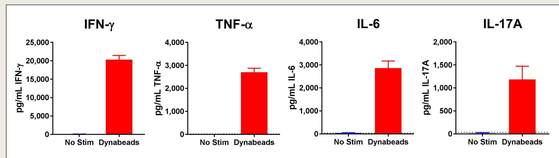
3 Probing IFN-γ-induced PD-L1 upregulation in HCC38 breast cancer cells



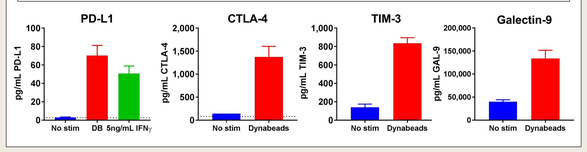


(A) IFN-γ-PD-L1 Cellular model for assessing bi-directional effects of cytokines and checkpoint molecules on immune and tumor cell populations. **(B)** HCC38 cells upregulate the expression of PD-L1 protein in response to IFN-γ treatment measured with AlphaLISA. **(C)** JAK pathway inhibitors, Tofacitinib (JAK3-specific) and Ruxolitinib (JAK1/2-selective), blocked the effect of overnight treatment with IFN-γ (125ng/mL) on upregulation of PD-L1 in 15,000 HCC38 cells in a dose-dependent manner. **(D)** JAK inhibitors also inhibit STAT1 phosphorylation at 2 sites in one hour of treatment with IFN-γ as assessed with AlphaLISA SureFire Ultra assays.

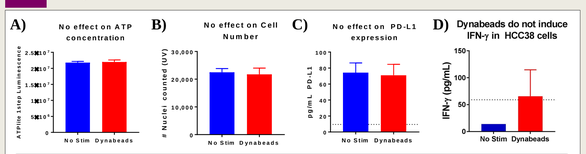
4 Effects of PBMC stimulation with Dynabeads on cytokine secretion and protein expression



PBMCs were cultured (at starting concentration of 25k cells/50 µL) with or without anti-CD3/CD28 Dynabeads (1bead/1cell ratio). After 4 days, supernatants were collected, cells were lysed and samples (*all from the same plate*) were assessed for expression of various **cytokines (Above)** and **immune checkpoint molecules (Below)** using AlphaLISA biomarker Assays (n=4 wells/condition, dotted line = assay LDL).

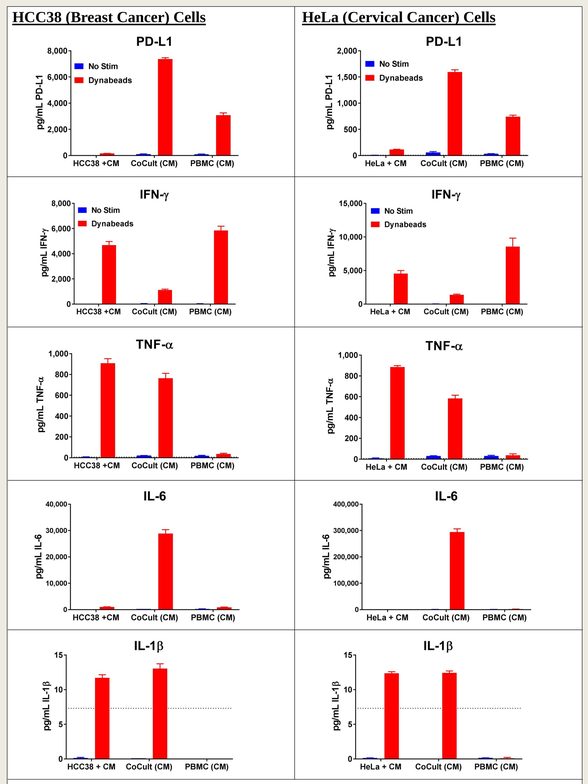


5 Dynabeads alone do not affect HCC38 cells



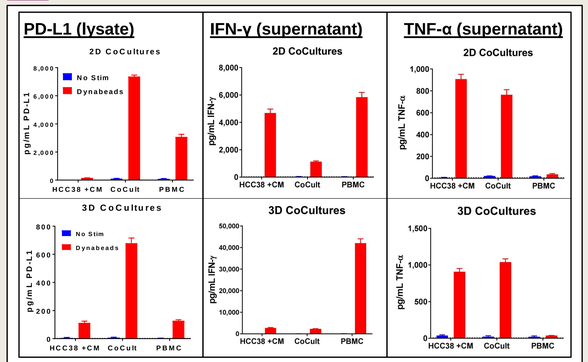
HCC38 cells seeded at 20,000 cells per well were treated with or without 20,000 Dynabeads for 2 days before cultures were assessed for viability/proliferation with ATPlite 1step **(A)** and by counting cells labeled with Hoechst using count nuclei function of the imaging module on the EnSight multimode plate reader **(B)**. No effects were observed on **(C)** PD-L1 expression levels (without IFN-γ added) and **(D)** IFN-γ secretion assessed with AlphaLISA(dotted line = assay LDL, n=10).

6 CoCulture and conditioned media effects on breast and cervical cancer cell lines



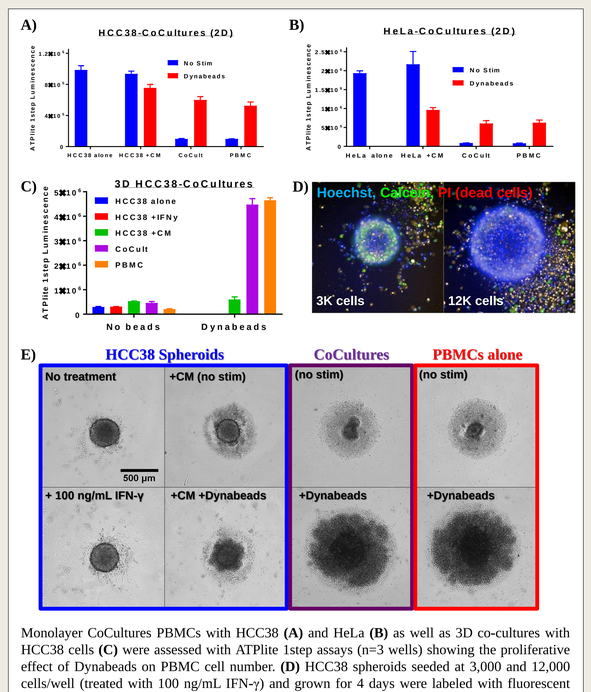
HCC38 (left panel) or HeLa cells (right panel) were grown for two days before addition of PBMC-conditioned media or PBMCs directly to wells which were either activated with Dynabeads (red bars) or not (blue bars). After two days of treatment, supernatants and lysates were collected and assessed for multiple proteins using AlphaLISA assay technology (n=3 wells/condition, dotted line = assay LDL).

7 3D vs 2D HCC38 Co-Cultures



HCC38 cells seeded at 25,000 in flat-bottomed plates and 5,000 cells per well in round-bottomed, ULA-coated microplates were grown for two days before addition of PBMC-conditioned media or PBMCs directly to wells . (Note – HCC38 spheroids form within 24 hours.) PBMCs had been either activated with Dynabeads (red bars) or not stimulated (blue).for 2 days. After two days of treatment, supernatants were collected, cultures were lysed, and 5µL samples (all from the same 2 plates) were assessed for multiple biomarkers using AlphaLISA assay kits. Results from three assays are shown here (n=3 wells/condition).

8 Treatment effects on viability, proliferation , and 3D morphology



Monolayer CoCultures PBMCs with HCC38 **(A)** and HeLa **(B)** as well as 3D co-cultures with HCC38 cells **(C)** were assessed with ATPlite 1step assays (n=3 wells) showing the proliferative effect of Dynabeads on PBMC cell number. **(D)** HCC38 spheroids seeded at 3,000 and 12,000 cells/well (treated with 100 ng/mL IFN-γ) and grown for 4 days were labeled with fluorescent dyes for nuclei (Hoechst), live cells (Calcein-AM), and dead cells (Propidium Iodide) and imaged on the Operetta (10X objective). These images illustrate how smaller spheroids are healthier and easier to image inside. **(E)** Brightfield Images (using a 4X objective) of 3D CoCultures of 5,000 HCC38 cells and PBMCs illustrate how IFN-γ, Conditioned Media (CM) and PBMCs activated with Dynabeads have different effects on Spheroid morphology.

9 Summary & Conclusions

- **PD-L1 is upregulated by IFN-γ treatment in HCC38 and HeLa cell cultures (cellular models of triple-negative basal breast cancer and cervical cancer)**
- **Stimulation of human PBMCs with anti-CD3/anti-CD28 Dynabeads results in cell expansion and upregulation of a variety of cytokines (IFN-γ, TNF-α, IL-6 & IL-17A) and immune checkpoint molecules (PD-L1, CTLA-4, TIM-3, & Galectin-9)**
- **Co-Culturing activated immune and tumor cell lines stimulates differential expression of some biomarkers compared to culturing with conditioned media**
- **More complex *in vitro* co-culture and 3D spheroid culture systems create more relevant experimental models for assessing intricate immune cell-tumor interactions**
- **AlphaLISA assays can be used to rapidly measure multiple (>10) proteins in supernatant and lysates from the same wells of a culture dish (from 5 µL samples)**
- **PerkinElmer's wide portfolio of reagents (AlphaLISA, ATPlite), microplates (ViewPlates, CellCarrier), plate readers (EnVision, EnSight), high content imagers (Operetta), and automation platforms simplify and optimize output from your cellular assays**

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