Measuring PD-L1 Expression in Breast Cancer Cell Lines with AlphaLISA

**Introduction**

In the tumor microenvironment, immune responses are weakened, directing immune cells to support tumorigenesis. Among the most promising approaches to activating therapeutic antitumor immunity is through the blockade of immune checkpoints. The programmed cell death-1 (PD-1) immune checkpoint pathway is a negative regulator of T-cell immune function. When PD-1 is bound to programmed cell death-ligand 1 (PD-L1), T cell response is suppressed. Many tumor cells escape anti-tumor immunity through their expression of Programmed Death Ligand 1 (PD-L1 or B7-H1), which interacts with T cell-expressed PD-1 and results in T cell apoptosis. PD-L1 expression has been studied in different cancers including kidney, lung, colorectal, pancreas, melanomas, head and neck squamous cell carcinomas, and breast cancer (He et al., 2015; Ritprajak and Azuma, 2015). In basal-like breast cancer, PD-L1 expression is associated with tumor severity and poor prognosis (Sabatier et al. 2014; Li et al. 2016). Basal-like breast tumors show a prominent lymphocytic infiltration and an ability to adapt to Interferon gamma (IFN-γ)-mediated responses (secreted by Type 1 helper T cells) to evade immunity via upregulation of PD-L1 protein (Karasar and Esendagli, 2014).
Several anti-PD-1 or PD-L1 monoclonal antibodies have been developed so far to treat a variety of cancers by blocking PD-1/PD-L1 complex formation. However, there remains a need for more robust, rapid, high-throughput assays to identify and qualify novel inhibitors of PD-1/PD-L1 binding and to detect both binding partners. We demonstrate here the utility and benefits of using AlphaLISA® assay technology for identifying and characterizing endogenous PD-L1 expression in cells. We show that PD-L1 is expressed and highly induced by IFN-γ in HCC38 cells, a basal B breast cancer cell line as compared to MCF-7 (luminal) cells. Further, the JAK/STAT pathway is shown to be crucial for this upregulation (see Figure 1A for an illustration of a cellular model of this pathway).

AlphaLISA technology allows for the detection of molecules of interest in a homogeneous, no-wash format. As illustrated in Figure 1B, a biotinylated anti-PD-L1 antibody binds to streptavidin-coated Donor beads while another anti-PD-L1 antibody is conjugated directly to AlphaLISA Acceptor beads. Both antibodies bind to PD-L1 and, when present, bring the Donor and Acceptor beads into close proximity of each other. Upon excitation at 680 nm, the Donor beads emit singlet oxygen molecules that travel in solution to activate the Acceptor beads, which then emit a sharp peak of light at 615 nm. This light emission can then be detected on an Alpha-enabled reader.

**Materials and Methods**

**Cell Culture and Treatment**

HCC38 (ATCC® CRL-2314™) or MCF-7 (ATCC® HTB-22™) cells were expanded in T-75 culture flasks (Corning, #430641U) and harvested by first rinsing with PBS (ThermoFisher, #14190250) and then using 0.25% Trypsin-EDTA (ThermoFisher, #25200056). Cells were seeded (50 μL/well) into PerkinElmer 96-1/2Area ViewPlates (#6005760) or 384-well ViewPlates (white, #6007480, or black-walled, #6007460) and allowed to attach and grow overnight. Cultures were then treated for 24 hours or more with recombinant human IFN-γ (BioLegend, #570206) diluted in culture media (25 μL added per well). In some experiments, treatment included a titration of JAK2- and JAK3-specific inhibitors (Ruxolitinib and Tofacitinib; Selleckchem #S1378 and #S2789 respectively) added just prior to IFN-γ addition.

**AlphaLISA Detection Assays**

For biomarker detection assays, culture media was aspirated and cells rinsed with HBSS (ThermoFisher #14025-134) prior to lysis. Cells were then lysed with 25 μL of 1X Immunoassay buffer (provided in kit or #AL000) for 30 minutes. Lysates were either used immediately or frozen at -20°C and thawed within two weeks for testing with AlphaLISA hPD-L1 detection assay (#AL344C). Samples (5 μL of lysates or standard curve) were added to a 384-well white OptiPlate (#6007290) and assays performed according to the kit manual. In short, a 20 μL mixture of 5 mg/mL anti-hPD-L1 antibody-conjugated AlphaLISA Acceptor beads and 50 μg/mL biotinylated anti-hPD-L1 in assay buffer was added to each well. After a one hour incubation period, 25 μL per well of 80 μg/mL streptavidin-coated Donor beads were added and plates incubated for another 30 minutes prior to reading the Alpha signal. The entire assay was run at room temperature. For most assays, plates were mixed slowly during the one hour incubation on a rotating shaker (DELFIA PlateShake, ~200 RPM), though this step is not necessary for good assay performance. The final assay volume is 50 μL but, as there is a high signal to background, this may be reduced by half or more to miniaturize the assay.

AlphaLISA SureFire® Ultra® p-STAT1 assays (#ALSU-PST1-A500 for Tyr701 and #ALSU-PST1-B500 for Ser727) were performed with 10 μL of lysates collected from a 10 minute lysis step with 1X SureFire Ultra Lysis buffer (provided in the kits) and the assay performed as prescribed in the manual for adherent cells with the two-plate protocol. In short, 5 μL of Acceptor Mix was added to samples and incubated for one hour before addition of 5 μL of Donor Mix. After a minimum one hour incubation (or up to overnight) at room temperature, plates may be read on an Alpha-enabled reader.

**AlphaLISA Signal Detection**

The AlphaLISA hPD-L1 assays and SureFire Ultra STAT1 assays were measured using a PerkinElmer EnVision® multimode plate reader (Figure 2A) using default values for standard Alpha detection. For high-throughput applications, when an even higher detection speed is required, the Alpha HTS module is recommended. The system incorporates unique temperature
control for sensitive Alpha and AlphaPlex™ assays, ensuring that results are both reproducible and accurate. In addition to fast, sensitive Alpha technology detection, the EnVision Multilabel microplate reader provides fluorescence intensity, luminescence, absorbance, fluorescence polarization, and time-resolved fluorescence detection technologies. The system is based on hybrid technology, combining filters and a monochromator for enhanced flexibility. It incorporates proprietary Direct Double Optics™ technology for high speed and sensitivity in simultaneous filter-based readouts, such as FRET assays, and for TRF, lamp-based excitation is standard, with the option of a high energy laser for higher speed and sensitivity.

**Standard Curve and Data Analysis**

Standard curves for the AlphaLISA PD-L1 assay were performed in the same diluent as the samples being tested (Immunoassay Buffer) using the recombinant standards provided in each kit. Curves were plotted in GraphPad Prism according to a nonlinear regression using the four-parameter logistic equation (sigmoidal dose-response curve with variable slope) and 1/Y² data weighting. The lower detection limit (LDL) of the assay was calculated by taking three times the standard deviation of the average of the background and interpolating off of the standard curve. PD-L1 protein levels in cellular lysates were quantitated by interpolation of AlphaLISA signal off the standard curve. Interpolated analyte concentrations represent the amount of protein in 5 μL of sample. All data shown (raw and interpolated signals) are the average of at least triplicate measurements.

**Fluorescent Staining, Imaging and Automated Cell Counting**

For cellular imaging and automated cell counting, cells were first stained with Hoechst 33342 (5 μg/mL; Life Technologies, #H3570) which fluoresces in the UV range. Images of each well were acquired using the cellular imaging module of the EnSight™ multimode plate reader (Figure 2B) using Brightfield and UV fluorescence filters. The EnSight multimode plate reader, alongside standard detection technologies, includes a cell-imaging option, which is provided by the well-imaging detection module. The cell imaging mode can be selected to suit your application: a fluorescence intensity mode with LED light source and filters for up to four colors (385/470/525/632nm), brightfield mode which is a fast, easy way to image cells without labelling, and digital phase contrast for imaging of non-labelled cells. In this application cell numbers were automatically counted using the count nuclei function.

For PD-L1 staining, HCC38 cells grown in black 384-well ViewPlates were fixed for 10 minutes with 10% formalin (VWR, #89370-094), rinsed three-four times by halves with PBS (Gibco, #10010-023), permeabilized and blocked with 0.1% Triton X-100 (Sigma, #93443) in 10% goat serum (Abcam #ab7481) in PBS for 30 minutes. They were then incubated overnight with anti-PD-L1 antibody (Sino Biologicals, #10084-R015) at 1:200 dilution in 1% Goat Serum. After rinsing three times, they were incubated for one hour in 1:200 of fluorescein (FITC)-conjugated Goat-anti-Rabbit IgG (H+L) from Jackson ImmunoResearch (#111-095-045) and 5 μg/mL Hoechst diluted in 1% goat serum. They were rinsed again three more times in PBS and imaged using the EnSight with the appropriate fluorescence filters.

**Results**

**AlphaLISA PD-L1 Detection in the HCC38 Breast Cancer Cell Line**

Interferon-γ is a potent multifunctional cytokine which is secreted primarily by activated NK cells and T cells and has been reported to greatly enhance PD-L1 expression in a subset of basal type breast cancer cells, including the HCC38 (basal B) cell line (Soliman H, et al. 2014). To assay for PD-L1 expression, HCC38 cells were plated in 96-well ½Area ViewPlates at seven different densities ranging from 15,000 cells titrated by halves down to 234 cells and grown overnight to allow adherence to the plate. This microplate was chosen initially because it allowed for testing (and imaging) a wider range of plating densities in a 96-well format while at the same time allowing the use of a smaller volume of lysis buffer. To confirm that IFN-γ treatment results in the upregulation of PD-L1 protein and to determine the optimal concentration to use, wells were treated the next day with four concentrations of IFN-γ diluted in media and added directly to wells. Cultures were lysed after two days in IFN-γ by first rinsing cultures with 1X HBSS and then lysing with 25 μL of Immunoassay buffer (which is provided in the kit) for 30 minutes on a plate shaker (DELFIA® Platsheake set at ~600-700 RPM).
Initially, different lysis buffers were tested, including AlphaLISA Lysis buffer (#AL003C) and Immunoassay buffer with added 0.1% Triton X-100. Though all buffers examined were effective, Immunoassay buffer alone was found to be sufficient for lysis and detection of PD-L1 in this cell line (data not shown). Lysis conditions should always be first optimized based on your sample of interest.

The data from our first experiment (Figure 3) show upregulation of PD-L1 within two days in response to IFN-γ treatment in HCC38 cultures. The standard curve generated from titrating human PD-L1 (Figure 3B) was used to interpolate the raw AlphaLISA signal (Figure 3A) to actual concentrations of PD-L1 protein in pg/mL (Figure 3C). The data show how increasing both cell numbers and concentrations of IFN-γ enhance PD-L1 expression in HCC38 cell cultures at all concentrations examined. The pronounced effect of IFN-γ on this cell line, combined with the high sensitivity of the AlphaLISA assay, resulted in a significant difference in expression even at the lowest IFN-γ concentration tested (1 ng/mL) in as few as 234 cells (Figure 3D). Also note that since the cultures were lysed with 25 μL of buffer and only 5 μL of lysate was used for the assay, the amount of sample engaged is actually 1/5th of the amount of cells plotted in all the graphs. Since the interpolated data (Figure 3C) show identical differences between experimental conditions as the raw data (Figure 3A), AlphaLISA signal may be plotted as an alternative to PD-L1 concentration.

Figure 3: HCC38 cell line expresses PD-L1 and expression is upregulated by IFN-γ treatment. Seven seeding densities of HCC38 cells were plated and treated the next day with different concentrations of IFN-γ. After two days of treatment, cells were lysed and assessed for PD-L1 expression and raw AlphaLISA signal plotted (A). B) Standard curve was generated from a 12-point half-log titration of human PD-L1 (freshly reconstituted). PD-L1 concentrations were interpolated from the raw data (A) using the standard curve (B) and presented in pg/mL (C). D) The lowest cell numbers showed significant effect of treatment with the smallest concentration of IFN-γ tested (dotted line = LDL = 0.6 pg/mL). All data points are an average of three wells.
PD-L1 Upregulation with IFN-γ Treatment and Cell Number

After showing how sensitive the assay is in detecting low levels of PD-L1 expression, we wanted to push the upper limits of expression further in our culture model by treating higher numbers of HCC38 cells with higher concentrations of IFN-γ and by increasing treatment time. To assess whether PD-L1 concentrations can be enhanced further, up to 30,000 seeded cells were treated with up to 500 ng/mL of IFN-γ and effects were compared after two days and three days of treatment. The data presented in Figure 4 illustrate how increasing treatment time and concentration affect PD-L1 expression and that these effects appear to be greater in cultures seeded with lower cell numbers.

PD-L1 Expression Compared in Two Breast Cancer Cell Lines

Previous studies have reported that PD-L1 expression varies in breast cancer tumors of different genomic subtypes and between breast cancer cell lines (Sabatier et al., 2014). To examine this further, both MCF-7 and HCC38 cells were plated

Figure 4. Optimizing PD-L1 upregulation with higher concentrations of cells and IFN-γ and examining effect of longer treatment. A) Seven different concentrations of cells starting from 30,000 were plated and treated the next day with four concentrations of IFN-γ. After two days (A) and three days (C), of treatment cells were lysed and assayed for PD-L1 expression. The effect of treatment time (two vs. three days) and concentration is examined more closely for the highest (D) and second lowest (B) cell numbers seeded.
(four densities) and treated with IFN-γ (four concentrations) for two days before measuring PD-L1 expression. The data in Figure 5 indicate that the MCF-7 cells (breast cancer cells of luminal origin) upregulate PD-L1 in response to IFN-γ treatment as well. However, MCF-7 cells expressed considerably less PD-L1 protein (compare raw data values between 5A and 5B) and the effect of IFN-γ resulted in a greater than 10-fold lower increase in expression compared to the five-fold increase in MCF-7 cells in Figure 5C and 5D). This observation is further supported in the literature by reports from flow cytometry experiments (Soliman et al. 2014).

**PD-L1 Upregulation by IFN-γ does not Correlate with Cell Number or Changes in Morphology**

One possible explanation for an observed upregulation of protein in response to treatment of cells in culture could be due to an increase in cell number. To rule out such an explanation and to determine if there is an effect of IFN-γ on cell numbers, we examined both the HCC38 cells and MCF-7 cells in culture by labeling with a nuclear stain (Hoechst) and imaging the wells using the imaging module of the EnSight multimode plate reader. This reader allows for rapid imaging of all the wells in a microplate (the whole well) and automatically selects for and counts the number cells in each well. The images were collected, analyzed and values automatically exported for assessment.

Graphs illustrating the results collected for four cell numbers seeded and four concentrations of IFN-γ are presented in Figure 6. The same wells that were imaged were also lysed and assessed for PD-L1 expression. The Hoechst stain was found not to interfere appreciably with AlphaLISA signal, so the wells that were imaged were also included in the average data presented in the Figure 5. The number of cells counted in each well after

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**Figure 5. IFN-γ effect on PD-L1 expression in different breast cancer cell lines.** MCF-7 and HCC38 cells were plated (four densities) and treated with IFN-γ (four concentrations) for two days before assessment with AlphaLISA for PD-L1 expression. A) MCF-7 cells upregulate PD-L1 in response to IFN-γ treatment but with an approximate 10-fold lower difference than the response seen in HCC38 cells (B). Data represented is average of six wells where half were stained with Hoechst for well imaging and automated cell counting.
two days of treatment (three total days in culture) indicated that IFN-γ treatment does not increase cellular proliferation and that very low levels of cell division are occurring in these cultures as observed in Figure 6B and 6C. This is especially obvious at the higher cell densities, where cell numbers observed are approximately equivalent to the number plated. To ascertain that we are seeing PD-L1 upregulation at the single cell level, we also examined expression with immunocytochemistry. HCC38 cells treated with and without IFN-γ were fixed and stained with Hoechst and an anti-PD-L1 antibody and fluorescein-labeled secondary antibody. Cultures were imaged on the EnSight and representative images (that are matched for

Figure 6. PD-L1 upregulation is not due to changes in cell numbers or viability as measured by automated cell counting with the EnSight Multimode plate reader. A) Shown here is a representative example of HCC38 cells stained with Hoechst and imaged in brightfield and UV filters (blue). The cell counting function automatically selects nuclei and is illustrated by the images on the right with orange outlines surrounding each cell nucleus. Results of the automated cell counting are graphed below for four concentrations of HCC38 cells (B) and MCF-7 cells (C) treated with four different concentrations of IFN-γ (n=3 wells).
contrast enhancement) are presented in Figure 7A and illustrate that upregulation of PD-L1 is occurring in individual cells. Additionally, to rule out any effect of IFN-γ on observable morphology, images were captured using brightfield parameters and the digital phase contrast option. Representative images, shown in Figure 7B indicate no obvious effects of treatment on cellular morphology.

**IFN-γ Effects on PD-L1 Upregulation Require the JAK1/2 Pathway**

IFN-γ signaling was previously reported to be regulated through the Interferon gamma receptor and JAK1/2 signaling (Bellucci et al. 2015; and illustrated in the model in Figure 1). To confirm that IFN-γ induces PD-L1 through the JAK pathway in our model system, two JAK pathway-specific inhibitors were tested for their ability to inhibit PD-L1 upregulation via IFN-γ. HCC38 cells were plated at 15,000 cells per well into white, 384-well ViewPlates and treated the next day with 12 concentrations of JAK inhibitor compound immediately followed by treatment with 125 ng/mL IFN-γ. Cultures were lysed and examined for PD-L1 expression with AlphaLISA and compound IC_{50} calculated and reported in

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**Figure 7.** PD-L1 expression upregulated by IFN-γ as shown by PD-L1 immunostaining cultures. A) HCC38 cell cultures were immunostained with Hoechst and PD-L1. Wells were either treated for two days with 200 ng/mL of IFN-γ (top images) or vehicle control (bottom images). B) Digital Phase Contrast (DPC) images of MCF-7 and HCC38 cells treated with either 500 ng/mL IFN-γ or vehicle collected on the EnSight. All wells shown here were seeded with 7500 cells.
Figure 8A. Tofacitinib is a selective JAK3 inhibitor with 20-fold less potency for JAK2. Ruxolitinib is specific for signaling through the JAK1/2 pathway, which is shown to be necessary for PD-L1 upregulation by IFN-γ. The JAK inhibitors do not appear to be cytotoxic as shown by no effect of Tofacitinib concentration on cell numbers counted (Figure 8B).

Downstream of JAK signaling, STAT1 has also been reported to play a role in PD-L1 upregulation (Meissl et al. 2017). To assess the involvement of STAT1 downstream in the JAK1/2 pathway in our system, the level of STAT1 phosphorylation was assessed in response to one hour treatment with JAK inhibitors and 200 ng/mL IFN-γ at two separate STAT1 phosphorylation sites (Tyrosine 701 and Serine 727) from lysate samples taken from the same well. To assess STAT1 phosphorylation, AlphaLISA SureFire Ultra p-STAT1 assays (#ALSU-PST1-A500 for Tyr701 and #ALSU-PST1-B500 for Ser727) were performed on 10 μL of samples prepared from 35 μL of lysis buffer added to each well for a 10 minute lysis step. The data from these experiments, graphed in Figure 8 (C and D), indicate that STAT1 phosphorylation at both Tyrosine 701 and Serine 272 is downregulated by JAK Inhibitors (in HCC38 cells stimulated with IFN-γ) within one hour with similar inhibition curves and IC₅₀ values as seen for PD-L1 protein expression after two days in the same inhibitors (Figure 8A).
Conclusions
Data presented here confirm that PD-L1 protein expression levels in human cells can be quickly and accurately assessed directly with the AlphaLISA human PD-L1 assay in a no-wash protocol. The data further illustrate that treatment of two breast cancer cell lines with IFN-γ is sufficient for inducing PD-L1 biomarker expression and that AlphaLISA technology can be used to assess differences in expression between cells from different tumor origins. Finally, PD-L1 upregulation via IFN-γ signaling is shown to be controlled through the JAK1/2 pathway and phosphorylation of STAT1 at Tyr701 and Ser727 is involved.

AlphaLISA assays provide a fast, powerful, homogeneous platform for screening potential inhibitors of PD-L1 expression in human cells. The technology has a number of distinct advantages including high signal to background, wide dynamic range, and an extremely simple, straight-forward protocol.

References