Introduction

Tumor cells have developed ways to evade the immune response by exploiting immune checkpoint pathways. One of these is the PD-1/PD-L1 (programmed cell death ligand 1) pathway. When PD-1, which is expressed on the T cell, binds to PD-L1 expressed on the tumor cell, the T cell response is suppressed. Utilization of this pathway leads to tumor immune escape and promotes tumor cell growth. In fact, PD-L1 expression increases with tumor severity in many types of cancer. Release of a soluble form of PD-L1 (sPD-L1) into circulation is one mechanism that tumors may use to evade the immune response; however, it is unclear whether sPD-L1 can bind PD-1 and deliver an inhibitory signal. Previous studies have shown that soluble forms of PD-L1 have been detected in supernatants of cancer cell lines. In addition, sPD-L1 has been detected in sera from cancer patients and higher levels of sPD-L1 have been associated with poorer prognosis. In another study, exposure of CD4+ and CD8+ T lymphocytes to tumor-derived sPD-L1 induced apoptosis. The regulatory mechanism of membrane-associated PD-L1 (mPD-L1) may be different from sPD-L1 and the biological role of the sPD-L1 has still not been fully elucidated.
In order to induce and upregulate the expression of soluble and membrane-associated PD-L1 in two cancer cell lines (HCC38 and H460), cells were co-cultured with activated PBMCs (peripheral blood mononuclear cells). PBMCs are a heterogeneous population of blood cells having round nuclei. They consist of monocytes, macrophages, dendritic cells and lymphocytes (T cells, B cells, and NK cells). We used Dynabeads®, polystyrene-coated microspheres with antibodies against human CD3 and CD28 proteins covalently coupled to the surface, to stimulate the activation and expansion of the T lymphocyte population within the PBMCs. The binding of the antibodies to CD3 and CD28 proteins on PBMCs mimics the in vivo effects of antigen presenting cells (APCs). This stimulates the cultured T cells to proliferate and further differentiate. Once activated, T cells can upregulate a variety of immune checkpoint molecules and secrete a number of different cytokines that can further induce PD-L1 expression on the tumor cell.7

Traditional methods for assessing soluble and membrane-associated PD-L1 are wash-based ELISA assays, which typically require 5-6 hours of assay time. AlphaLISA® technology provides a rapid, no-wash bead-based alternative to traditional ELISAs. Figure 1 shows an example of an AlphaLISA assay setup used to detect PD-L1. Streptavidin Donor beads bind a biotinylated anti-PD-L1 antibody and an anti-PD-L1 antibody is directly conjugated to the AlphaLISA Acceptor beads. When PD-L1 is present in the sample, the antibodies bind and bring the Donor and Acceptor in close proximity. Upon excitation at 680 nm, the Donor beads generate singlet oxygen which can activate the AlphaLISA Acceptor beads, resulting in light production at 615 nm. In the absence of PD-L1, no signal is generated.

Add 5 µL of sample or 5 µL of analyte standard dilution

Add 20 µL of 2.5X Mix (freshly prepared) AlphaLISA Anti-hPD-L1 Acceptor Beads (10 µg/µL final) and Biotinylated Anti-hPD-L1 antibody (0.15 µg/µL final)

Incubate 60 minutes at 23 °C

Add 25 µL of 2X Streptavidin Donor Beads (40 µg/mL final)

Incubate 30 minutes at 23 °C (subdued lighting)

Read plate on EnVision plate reader

Figure 2. Assay protocol for AlphaLISA PD-L1 detection. Results are obtained in less than 2 hours.

Materials and Methods

Cell Culture
The cell lines HCC38 (ATCC®, #CRL-2314™) and H460 (ATCC®, #HTB-177™) were maintained in 75 mL cell culture flasks (Corning, #430641U) in RPMI-1620 (ATCC, #30-2001) supplemented with 10% FBS (ThermoFisher, #26140-079) and typically passaged twice per week. Cells were counted and resuspended at a density of 250K/mL (HCC38 cells) or 400K/mL (H460 cells). 3 mL of HCC38 cells (750,000 final) were plated in four T25 flasks (Corning, #353108) and 3 mL of H460 cells (1.2 million cells per flask) were plated in three T25 flasks. Normal human primary PBMCs (ATCC®, #PCS-800-011™) stored in liquid nitrogen were rapidly thawed, transferred to ice-cold Hanks Buffered Salt Solution (HBSS, ThermoFisher, #14025-134), and counted. Cells were spun down, supernatant removed, and cells were resuspended in RPMI + 10% FBS at 1.5 million cells/mL. Next, 1 mL of PBMCs in suspension was added to each of two flasks of HCC38 and two flasks of H460 cells. Human T-activator CD3/CD28 Gibco Dynabeads® (ThermoFisher, #11131D) were washed and resuspended in culture media at a concentration of 1.5 million/mL (one bead to one PBMC ratio) and 1 mL was added to one of the HCC38 + PBMCs flasks, one of the H460 + PBMCs flasks, and the flask of PBMCs alone. Finally, to one flask of HCC38 cells, 100 ng/mL of IFNγ (BioLegend, #570206) was added. All flasks were brought up to a final volume of 5 mL with RPMI + 10% FBS media. Cells were incubated for four days at 37 °C.

To prepare samples for testing, supernatants were removed from the flasks and placed into a 15 mL tube and centrifuged at 1000 rpm for five minutes. After spinning, to be sure that all cells were removed, supernatants were collected and added to new 15 mL tubes and centrifuged a second time. Finally, the supernatants were removed and added to a fresh 15 mL tube. After removing the supernatant, 5 mL of 1X Immunoassay buffer (supplied in the AlphaLISA PD-L1 kit) was added to each flask and incubated for 10 minutes. The 5 mL of lysates were removed from the flask after vigorously pipetting and transferred to a 15 mL conical tube.

PD-L1 AlphaLISA Assay
All assays were run using the AlphaLISA human PD-L1 kit (PerkinElmer, #AL355C) according to the manual. The assay protocol is shown in Figure 2. Briefly, 5 µL of each supernatant and lysate sample was pipetted into wells of an AlphaPlate™-384 (PerkinElmer, #6005350) microplate in triplicate. Next, 20 µL of a 2.5X mixture of biotinylated anti-PD-L1 and PD-L1 conjugated AlphaLISA Acceptor beads was added and the mixture incubated for one hour. Finally, 25 µL of 2X Streptavidin Donor beads was added and incubated for 30 minutes. For the standard curves, the recombinant PD-L1 was diluted in either 1X Immunoassay buffer (IAB) for quantification of lysates, RPMI + 10% FBS for quantification of supernatants, or a 1:1 ratio of IAB to RPMI/10%FBS for PBMC + Dynabead® lysates.
Instrumentation and Data Analysis
All assays were measured on the EnVision® 2015 multimode plate reader. The data were analyzed using GraphPad Prism® version 7 software. The binding curves were generated using nonlinear regression (assuming there is one binding site and none of the signal comes from non-specific binding), using a 4-parameter logistic equation (sigmoidal dose-response curve with variable slope). The lower detection limits were calculated by interpolating the average of the background plus three times the standard deviation of the background onto the standard curves.

Results and Discussion
Soluble PD-L1 is Upregulated in HCC38 Cells After Co-Culture with Activated PBMCs
The membrane associated form of PD-L1 is detectable and shown to be upregulated in a variety of cancer cell lines; however it is unclear the exact role of the soluble form of PD-L1. To be certain that only the soluble form of the protein was measured, supernatants were removed and centrifuged twice to remove any cells remaining. The AlphaLISA counts measured for each sample were interpolated from the standard curves shown in Figure 3. For supernatants, the standard curve run in RPMI + 10% FBS was used for interpolation. For lysates, the standard curve run in Immunoassay buffer was used for interpolation, with the exception of the PBMCs + Dynabead® lysates, which were interpolated from the standard curve in RPMI/10% FBS + IAB (1:1). This was due to the fact that the PBMCs were in suspension and therefore, not all media was removed prior to lysis.

Here we observed that prior to the interaction with T cells, the amount of sPD-L1 was not detectable in the supernatants of HCC38 cells (Figure 4A). As a control, the cells were incubated with non-activated PBMCs, and again no sPD-L1 was detected. Previous studies have shown that IFNγ can induce expression of mPD-L1 in HCC38 cells.8 We saw that 100 ng/mL of IFNγ induced expression of low levels of the sPD-L1 in HCC38 cell supernatants. It is unclear whether the soluble form detected here is a cleaved form of the membrane bound PD-L1 or if it is an alternatively spliced form of PD-L1 that is secreted. Interestingly, the activation of PBMCs alone using Dynabeads® also showed detectable levels of sPD-L1 in the supernatants. However, in the condition where activated PBMCs are co-cultured with the HCC38 cells, the increase in sPD-L1 expression exceeded what would be expected from simply combining the amounts of sPD-L1 generated by activated PBMCs and HCC38 cells cultured alone. The Dynabeads® themselves had no effect on PD-L1 expression in HCC38 cells alone (data not shown). This suggests that direct interaction between the activated T cell population in the PBMCs and the HCC38 cells results in a significant increase in the soluble form of PD-L1 secreted into the supernatants.

We also looked at the mPD-L1 levels in the lysates of the HCC38 cells. After the supernatants were removed, the adherent cells were lysed and the levels of PD-L1 were measured. Low levels of the mPD-L1 were detectable in the lysates of the HCC38 cells (Figure 4B) and low levels of mPD-L1 were also detected in the presence of non-activated PBMCs. We confirmed that 100 ng/mL of IFNγ induces expression of mPD-L1. We see that co-culturing resulted in a significant increase in mPD-L1 expression (~124-fold) compared with the isolated HCC38 cells.

Soluble PD-L1 is Upregulated in H460 Cells After Co-Culture with Activated PBMCs
H460 cells were also co-cultured with activated or non-activated PBMCs to measure expression levels of both sPD-L1 and mPD-L1. As shown in Figure 5A, the cells alone produced low, but detectable, levels of sPD-L1. This is consistent with previous studies that showed sPD-L1 could be detected in the supernatants of H460 cells.4 With the addition of PBMCs, there was no significant change in the levels of sPD-L1; however, co-culturing with activated PBMCs resulted in a significant increase in the levels of sPD-L1 secreted. This again exceeded the levels that would result from the addition of the sPD-L1 from activated, isolated PBMCs and isolated H460 cells. Interestingly, the increase in the mPD-L1 was not as dramatic for the co-culture of activated PBMCs and H460 cells (Figure 5B) as was seen for the HCC38 cells. There was an increase in the mPD-L1, but the increase was only about 1.7-fold over the mPD-L1 from isolated H460 cells. The increase in sPD-L1 was ~3-fold over the amount obtained by the addition of PD-L1 from isolated H460 cells and from activated isolated PBMCs. This suggests that the mechanism by which activated PBMCs promote an increase sPD-L1 secreted from H460 cells may be different than the mechanism by which mPD-L1 increases.
Figure 4. Comparison of expression levels of sPD-L1 (A) in supernatants and mPD-L1 (B) in lysates of HCC38 cells.

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<th>sPD-L1 (pg/mL)</th>
<th>mPD-L1 (pg/mL)</th>
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<tr>
<td>HCC38 Cells</td>
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</tr>
<tr>
<td>HCC38 + PBMCs</td>
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<td>HCC38 + IFNγ</td>
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Figure 5. Comparison of expression levels of sPD-L1 (A) in supernatants and mPD-L1 (B) in lysates of H460 cells.

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Figure 4. Comparison of expression levels of sPD-L1 (A) in supernatants and mPD-L1 (B) in lysates of HCC38 cells.
Conclusions

In this application note, we showed the detection and quantification of both soluble PD-L1 in supernatants of cells and membrane-associated PD-L1 in cell lysates utilizing AlphaLISA technology. We showed how two different cell lines, when co-cultured with activated PBMCs, significantly increased the amount of soluble PD-L1 in the supernatants. In both cases, the amount detected was more than the addition of sPD-L1 from activated isolated PBMCs and that of isolated cells. We also compared the changes in levels of sPD-L1 to that of the mPD-L1 in cellular lysates and showed differing patterns of expression between the two cell lines with and without activation of the PBMCs. H460 cells had a higher background level of mPD-L1 in the lysates but had a smaller increase when co-cultured compared with the HCC38 cells. AlphaLISA assays provide a quick and easy way to measure immune checkpoint molecules with faster time to results compared to traditional wash-based ELISAs.

References