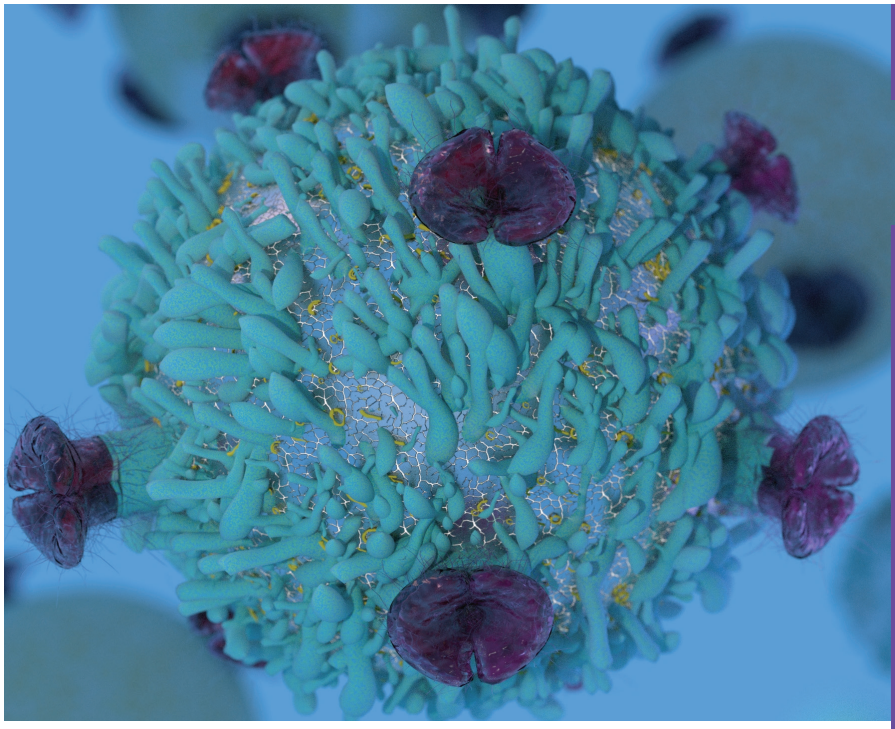

APPLICATION NOTE

LANCE TR-FRET

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Measuring PD-L1 and PD-1 Expression in Human Cells with LANCE *Ultra* TR-FRET

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).

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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 assays to detect expression levels of both binding partners. Here, we demonstrate the utility and benefits of using LANCE® *Ultra* TR-FRET assay technology for identifying and characterizing endogenous PD-L1 and PD-1 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, in comparison to significantly lower levels in 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). Finally, we show that LANCE can be used to quantify PD-1 expression in lysates produced from a cell line that overexpresses PD-1.

LANCE time-resolved fluorescence resonance energy transfer (TR-FRET) technology allows for the detection of molecules of interest in a homogeneous, no-wash format. These assays utilize the unique fluorescent properties of a europium-based chelate (Eu chelate) as the donor. Eu chelates have high quantum yield, large Stokes shift, a narrow-banded emission, and a long lifetime. These properties make Eu chelates ideal energy donors in TR-FRET assays, as they are less susceptible to interference and reduce background signal. U*Light*™ is a small, light-resistant acceptor dye that has a red-shifted emission maximum at 665 nm.

Using LANCE *Ultra* technology, PerkinElmer has developed sensitive immunoassay kits to measure both PD-L1 and its receptor, PD-1 in a variety of samples. In the LANCE PD-L1 assay, illustrated in Figure 1B, human PD-L1 protein is bound by both a Europium-labeled-anti-PD-L1 antibody and U*Light*-labeled-anti-PD-L1 antibody bringing the Europium and U*Light* into close proximity of each other. Upon excitation at 320 or 340 nm, the Europium activates the U*Light* through fluorescence energy transfer (FRET) to emit light at 665 nm. This light emission can then be detected on a TR-FRET enabled reader. The experiments described here illustrate how you can use LANCE technology to assay PD-L1 and PD-1 protein expression in human cell culture models.

Materials and Methods

Cell Culture and Treatment

HCC38 (ATCC® CRL-2314™) or MCF-7 (ATCC® HTB-22™) cells were seeded (50 µL/well) into PerkinElmer 96-well ½Area 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.

LANCE Detection Assays

For detecting human PD-L1 protein expression from cells in culture, media were aspirated and cells rinsed with HBSS (ThermoFisher #14025-134) prior to lysis. Cells were then lysed with 25 µL of 1X LANCE *Ultra* HiBlock buffer for 30 minutes. Please note that 2 mL of 5X buffer is provided in the assay kit and to order additional buffer for lysing cells, use catalog number TRF1011C for 10 mL (or TRF1011F for 100 mL). Lysates were either used immediately or frozen at -20 °C and thawed within two weeks for testing with LANCE hPD-L1 detection assay (#TRF1355). Samples (15 µL of lysates) were added to a 384-well white OptiPlate (#6007290) and assays performed according to the kit manual. In short, 2.5 µL of 2.4 nM Europium-labeled anti-hPD-L1 antibody diluted in assay buffer (0.3 nM in final assay volume) was added to each well and the plate was incubated for 30 minutes at room temperature. This was followed by 2.5 µL per well of 24 nM U*Light*-labeled anti-hPD-L1 antibody (3 nM final) to make an assay volume of 20 µL. After a two hour incubation period, plates were read on an EnVision® using standard LANCE settings with both laser and flashlamp excitation (though most data reported here was from laser excitation). For most assays, plates were mixed slowly during incubation periods on a rotating shaker (DELFIA® PlateShake, ~200 RPM), though this step is not necessary. For all-in-one well assays, HCC28 cells were plated in 384-well CulturPlates (#6007680), media was removed, 15 µL of *Ultra* HiBlock buffer was used to lyse cells, and the rest of the assay was performed directly in the plate.

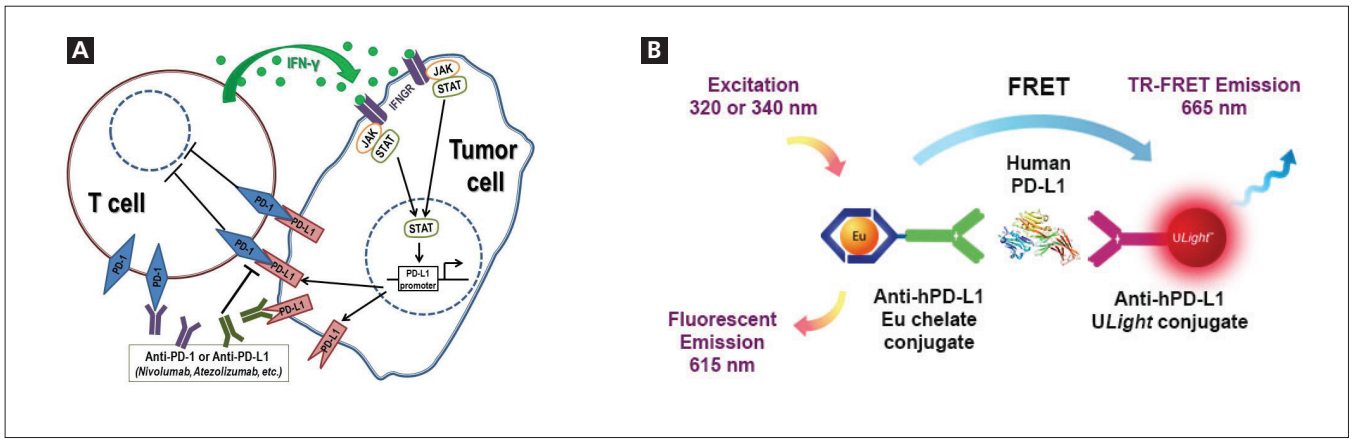

Figure 1. (A) Cellular model of the PD-1/PD-L1 pathway interaction between immune and tumor cells and (B) LANCE *Ultra* TR-FRET hPD-L1 detection assay schematic.

For LANCE assay detection of human PD-1, cellular lysates were purchased from RayBiotech (#230-10198). The PD-1-positive lysates were generated from HEK293 cells transfected with expression vectors harboring the PD-1 gene. Lysates were produced by cell harvesting and washing in ice-cold PBS with mammalian cell protease inhibitor cocktail and lysed further with freeze-thaw cycles. The LANCE PD-1 assay (#TRF1343) was run as described in the kit manual on 15 µL samples alongside a standard curve in order to quantify actual amounts of protein. Lysates from HEK293 cells that were transfected with an empty expression vector were tested alongside as a negative control.

LANCE Signal Detection

The LANCE hPD-L1 assays were measured using a PerkinElmer EnVision® multilabel plate reader (Figure 2A) equipped with a laser using default values for TR-FRET detection for both laser and flashlamp excitation. In addition to fast, sensitive LANCE technology detection, the EnVision Multilabel microplate reader provides fluorescence intensity, luminescence, absorbance, fluorescence polarization, and AlphaLISA® and AlphaPlex™ assay 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 LANCE PD-L1 assay were performed in the same diluent as the samples being tested (LANCE *Ultra* HiBlock 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 $1/Y^2$ data weighting. The lower detection limit (LDL) of the assay was calculated by taking two times the standard deviation of the average of the background and interpolating off of the standard curve. PD-L1 and PD-1 protein levels in cellular lysates were quantitated by interpolation of LANCE signal off the standard curve. Interpolated analyte concentrations represent the amount of protein in 15 µL of sample. All data shown are the average of at least triplicate measurements.

Cellular Fluorescent Staining, Imaging and Automated Cell Counting

For cellular imaging and automated cell counting, cells were first stained with Hoechst 33342 (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 (385/470/525/632 nm), 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 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.

Figure 2. (A) EnVision multilabel plate reader and (B) EnSight multimode plate reader.

Results

LANCE 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 while also using a smaller volume

of lysis buffer than a standard 96-well plate format. 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. Cultures were lysed after two days in IFN-γ by first rinsing cultures with 1X HBSS and then lysing with 25 µL of LANCE *Ultra* HiBlock Buffer for 30 minutes on a plate shaker (DELFIA Plateshake set at ~600-700 RPM). Lysates (5 µL/well) were then transferred to an assay plate to be run alongside a titration of human PD-L1 to generate a standard curve.

LANCE TR-FRET assays can be measured on the EnVision using either Laser or Flashlamp excitation and the signal is extremely stable, often lasting for many days and can be re-read multiple times without significant loss of signal. For our experiments, the laser excitation was found to be a little more sensitive when analyzing the standard curves shown in Figure 3. Additionally, the ratio of the 665 nm signal (from U*Light*) normalized to the 615 nm (Europium) signal was used for all experiments, though the 665 nm signal may be used if the appropriate filters are not available to measure at both wavelengths.

The standard curve (Figure 3) was used to interpolate the LANCE signals (Figure 4A) generated from samples to concentrations of PD-L1 protein in pg/mL (Figure 4B). The data from this first cellular experiment show upregulation of PD-L1 response to IFN-γ treatment within two days in HCC38 cultures. The data illustrate how increasing both cell numbers and concentrations of IFN-γ enhance PD-L1 expression in HCC38 cell cultures. The pronounced effect of IFN-γ on this cell line, combined with the high sensitivity of the LANCE assay, resulted in a noticeable difference in even the lowest number of cells tested (234 cells). Since the cultures were lysed with 25 µL of buffer and only 15 µL of lysate was used for the assay, the amount of sample engaged is only a fraction of the amount of cells plotted in all the graphs. Since the interpolated data (Figure 4B) show similar patterns between the experimental conditions and the raw data (Figure 4A), the LANCE ratiometric signal data may be used as an alternative to PD-L1 concentration to directly compare different treatment conditions in the same experiment.

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 determine the upper limits of detection 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 expression levels can be enhanced further, up to 30,000 seeded cells were treated with up to 500 ng/mL of IFN-γ and the effects were compared after two days and three days of treatment. The data presented in Figure 5 illustrate how increasing IFN-γ treatment time and concentration affect PD-L1 expression and that these effects appear to be greater in cultures seeded with lower cell numbers.

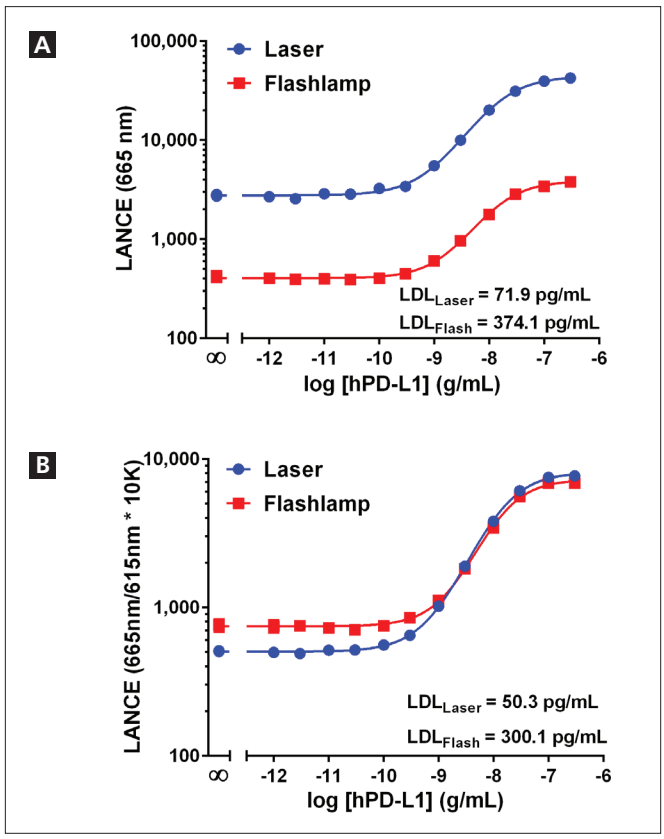

Figure 3. PD-L1 standard curves read on the EnVision using different excitation sources (A & B) and measurement methods (A vs. B). The LDLs calculated for two different excitation sources (laser and flashlamp) and detection methods (615 nm signal alone or normalized signal) are reported on each graph. All data points are an average of three wells.

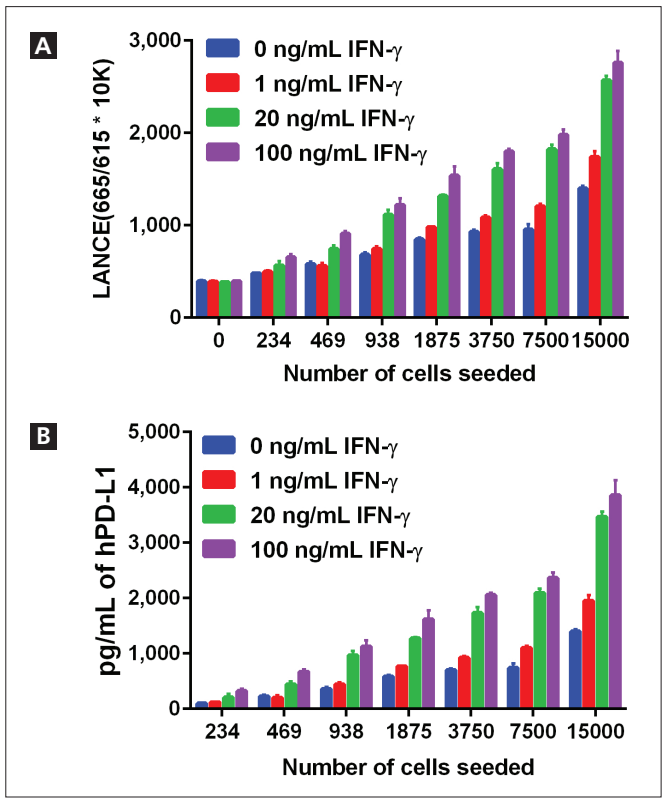

Figure 4. HCC38 cell line expresses PD-L1 and expression is upregulated by IFN-γ treatment. Seven seeding densities of HCC38 cells were plated and treated the next day with different concentrations of IFN-γ. After two days of treatment, cells were lysed and assessed for PD-L1 expression. A) LANCE raw data is plotted as the ratio of fluorescence signals at 665 nm/615 nm multiplied by 10,000. B) Concentrations of PD-L1 were interpolated from the raw data on a standard curve and presented in pg/mL.

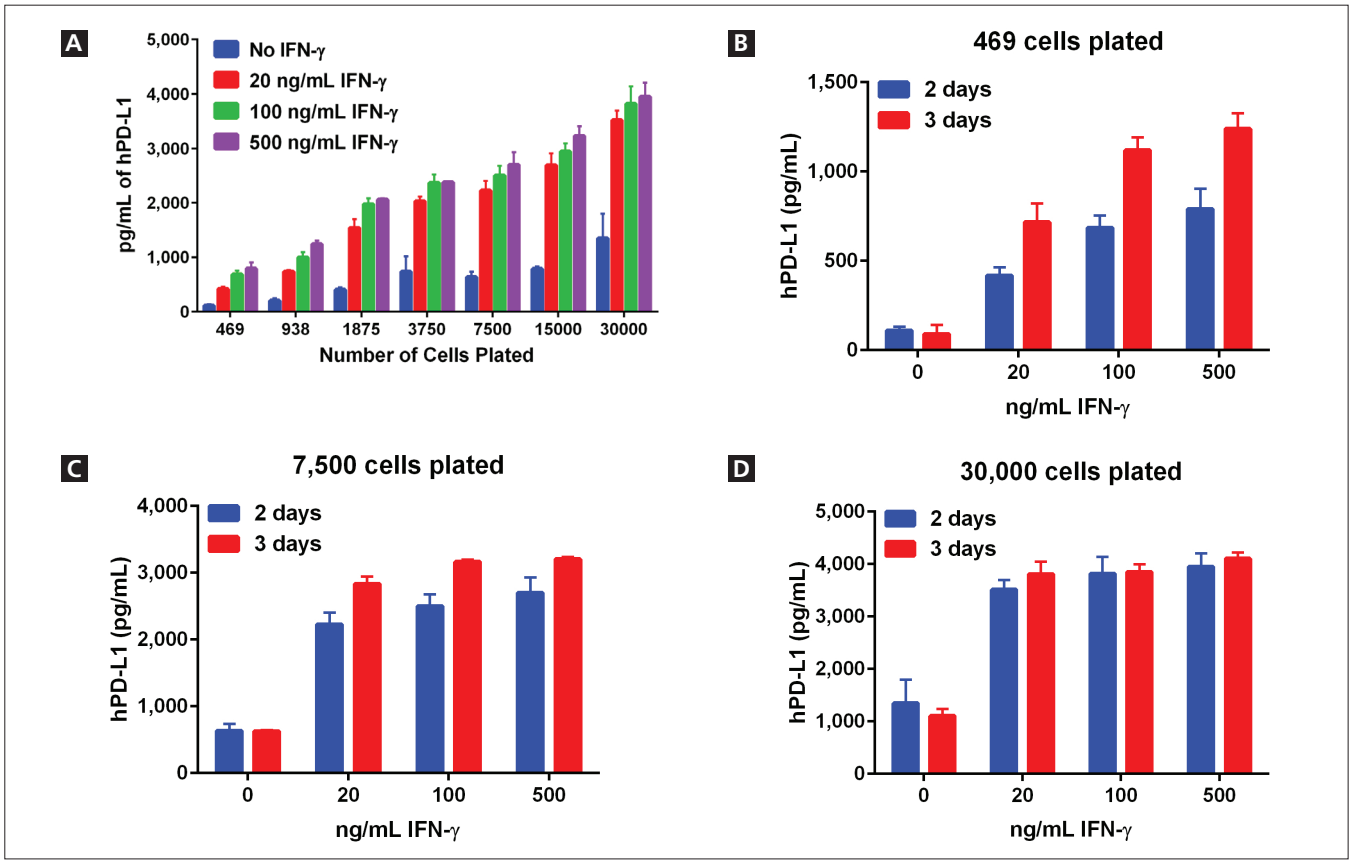

Figure 5. Optimizing PD-L1 upregulation with higher concentrations of cells and IFN-γ and examining effect of longer treatment. A) Seven different concentrations of cells starting from 30,000 were plated and treated the next day with four concentrations of IFN-γ. After two days and three days of treatment, cells were lysed and assayed for PD-L1 expression. All data for the two day treatment is shown in A. The effect of treatment time (two vs. three days) and concentration is examined more closely for three different concentrations of cells seeded in graphs B, C and D.

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, MCF-7 and HCC38 cells were plated (at three densities) and treated with (four concentrations of) IFN- γ for two days before measuring PD-L1 expression. The data in Figure 6 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 and the effect of IFN-γ resulted in a much lower increase in expression in MCF-7 cells compared to the response seen in HCC38 cells (compare 6A and 6B). This observation is further supported in the literature by reports from flow cytometry experiments (Soliman *et al.* 2014).

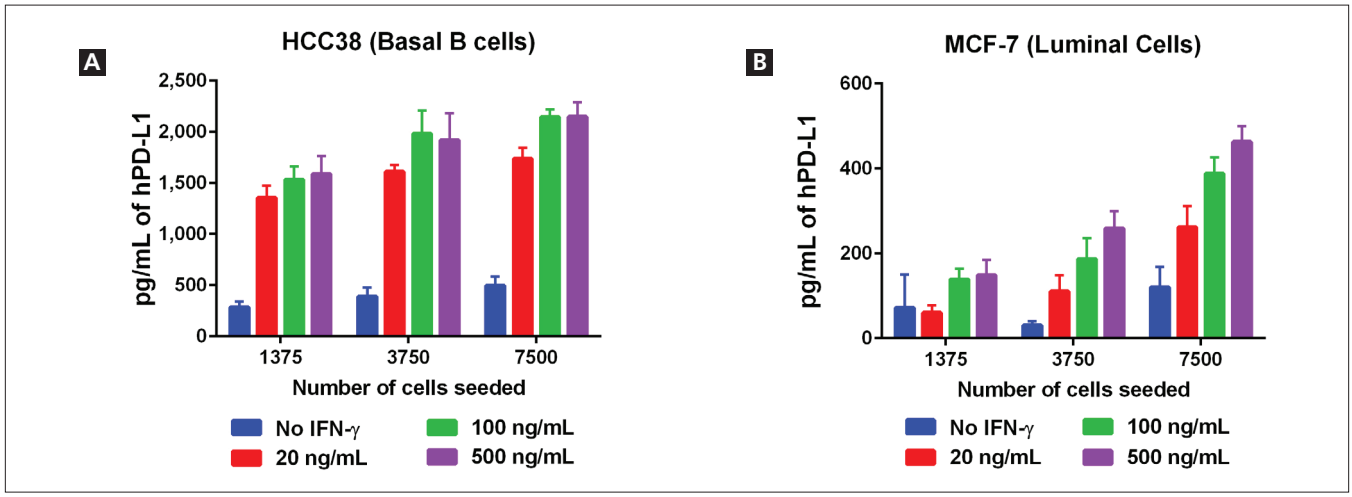

Figure 6. IFN-γ effect on PD-L1 expression in different breast cancer cell lines. MCF-7 and HCC38 cells were plated (four densities) and treated with IFN-γ (three concentrations) for two days before assessment with LANCE for PD-L1 expression. A) MCF-7 cells upregulate PD-L1 in response to IFN-γ treatment but with an approximately 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.

Increased PD-L1 Expression 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 increased cell proliferation after treatment. To rule out such an explanation and to determine if there is an effect of IFN-γ on cell proliferation, 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 three cell numbers seeded and four concentrations of IFN-γ are presented in Figure 7. 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 LANCE signal, so the wells that were imaged were also included in the average data presented in the Figure 6. The number of cells counted in each well after two days of treatment (three total days in culture) indicated that IFN-γ treatment does not increase cellular proliferation (Figure 7B and 7C) and that very low levels of cell division are occurring. This is especially obvious for HCC38 cells at the higher densities, where cell numbers observed are approximately equivalent to the number plated (Figure 7B).

To ascertain whether the data reflect 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 with a fluorescein-labeled secondary antibody. Cultures were imaged on the EnSight and the representative images (that are matched for contrast enhancement) are presented in Figure 8A 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 8B, indicate no obvious effects of treatment on cellular morphology in either cell line.

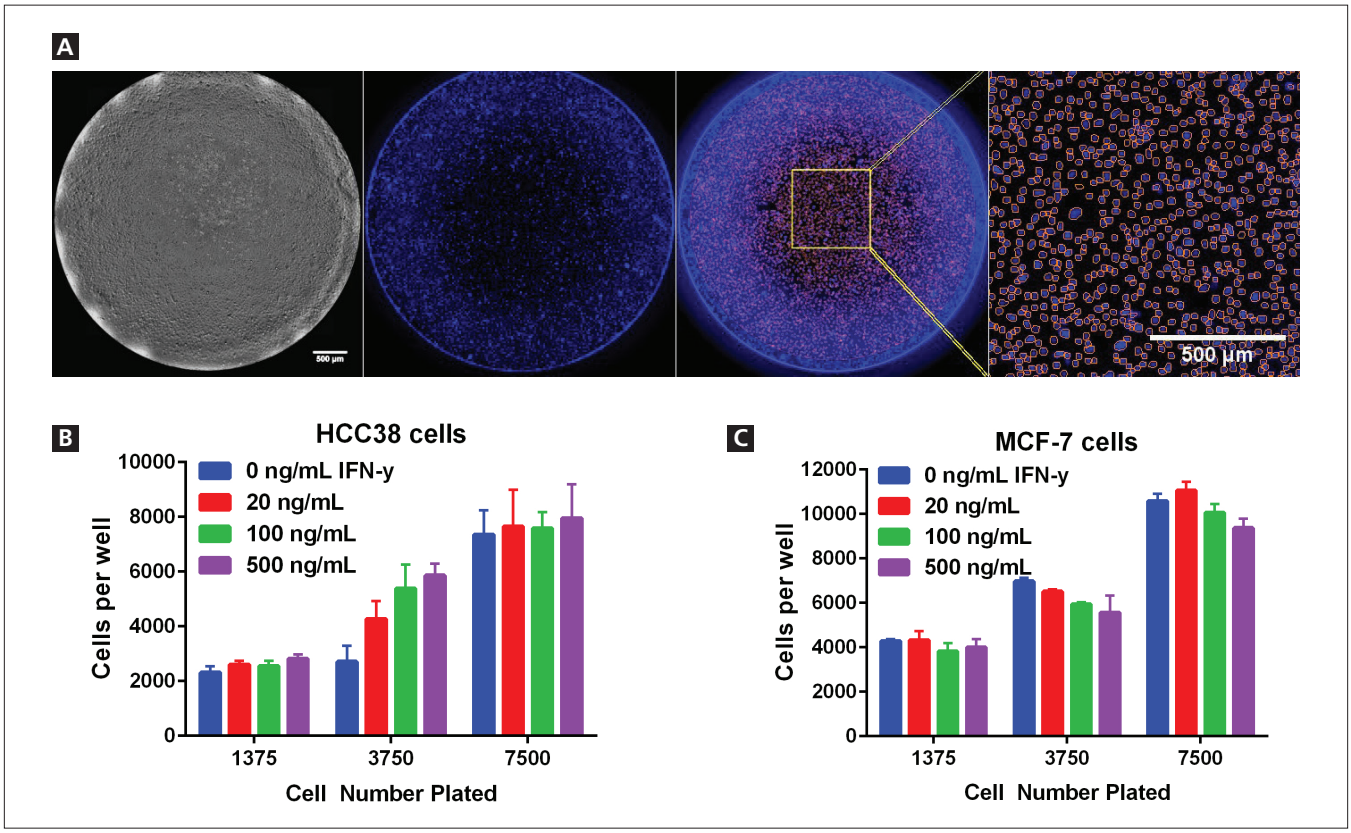

Figure 7. PD-L1 upregulation is not due to changes in cell numbers or viability as measured by automated cell counting with the EnSight Multimode plate reader. A) Shown here is a representative example of HCC38 cells stained with Hoechst and imaged in brightfield and UV filters (blue). The cell counting 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 three concentrations of HCC38 cells (B) and MCF-7 cells (C) treated with 4 different concentrations of IFN-γ (n=3 wells).

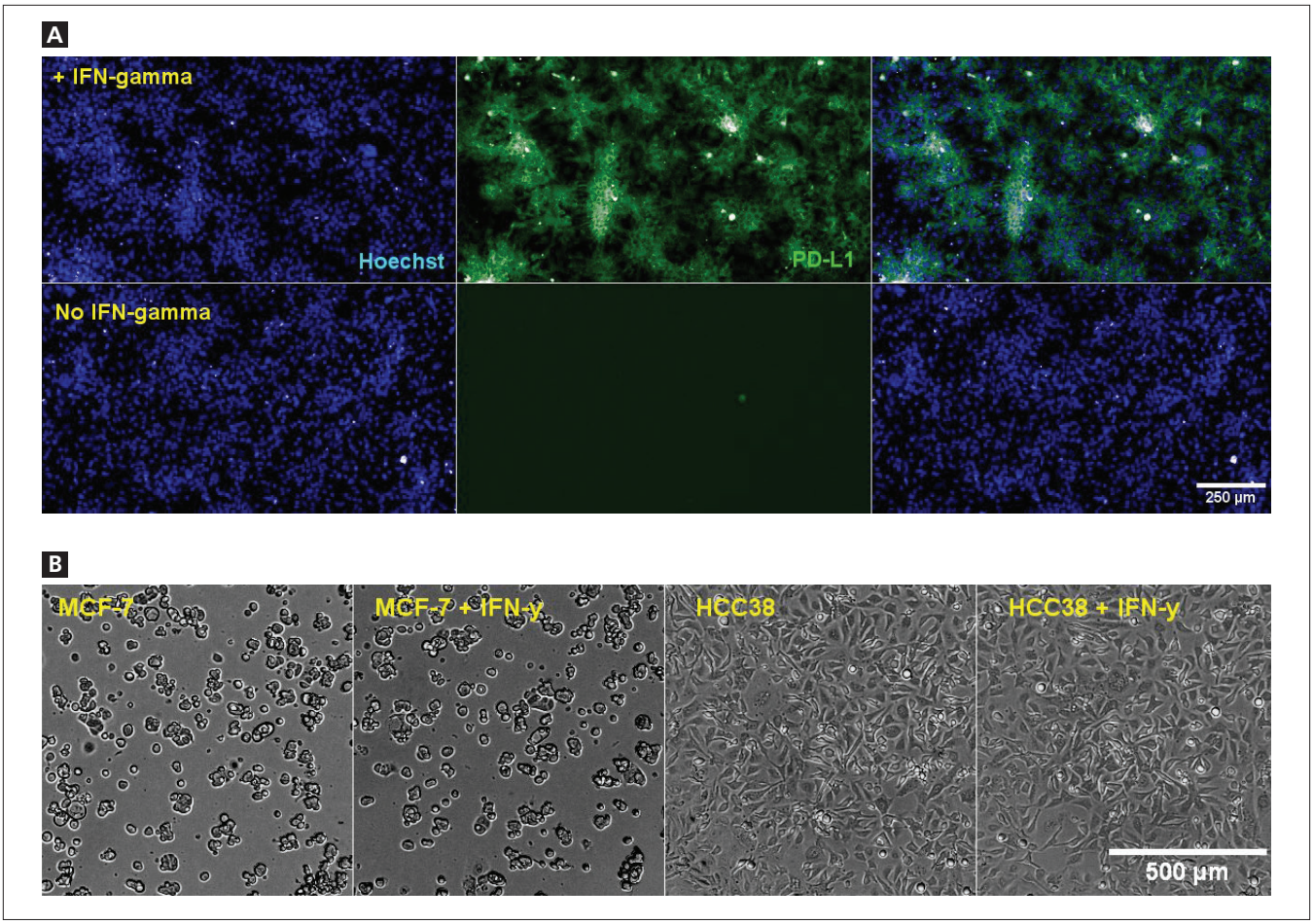

Figure 8. Upregulation of PD-L1 expression by IFN-γ shown by PD-L1 immunostaining. A) HCC38 cell cultures were immunostained with Hoechst and anti-PD-L1 antibody. Wells were either treated for two days with 200 ng/mL of IFN-γ (top images) or vehicle control (bottom images). B) Digital Phase Contrast (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.

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, the JAK pathway-specific inhibitor Tofacitinib was tested for the ability to inhibit PD-L1 upregulation via IFN-γ. HCC38 cells were plated at 15,000 and 30,000 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 100 ng/mL IFN-γ. Cultures were lysed, 15 µL samples removed and examined in 384-well OptiPlates for PD-L1 expression and compound IC_{50}s calculated and reported in Figure 9A. Tofacitinib is a selective JAK3 inhibitor with 20-fold less potency for JAK2. The JAK inhibitors do not appear to be cytotoxic, as shown by no effect of Tofacitinib concentration on cell numbers counted on the EnSight (Figure 9B). To further tease out whether the pathway for IFN-γ signaling is through JAK3 or JAK2, the inhibitor Ruxolitinib, which is specific for inhibition of signaling through the JAK1/2 pathway, was examined alongside Tofacitinib in 15,000 cells. The assay was run in the same format as done previously (Figure 9C) as well as in an all-in-one-well format in 384-well CulturPlates (opaque white plates) with the data presented in Figure 9D. These data suggest preferential involvement of the JAK1/2 pathway.

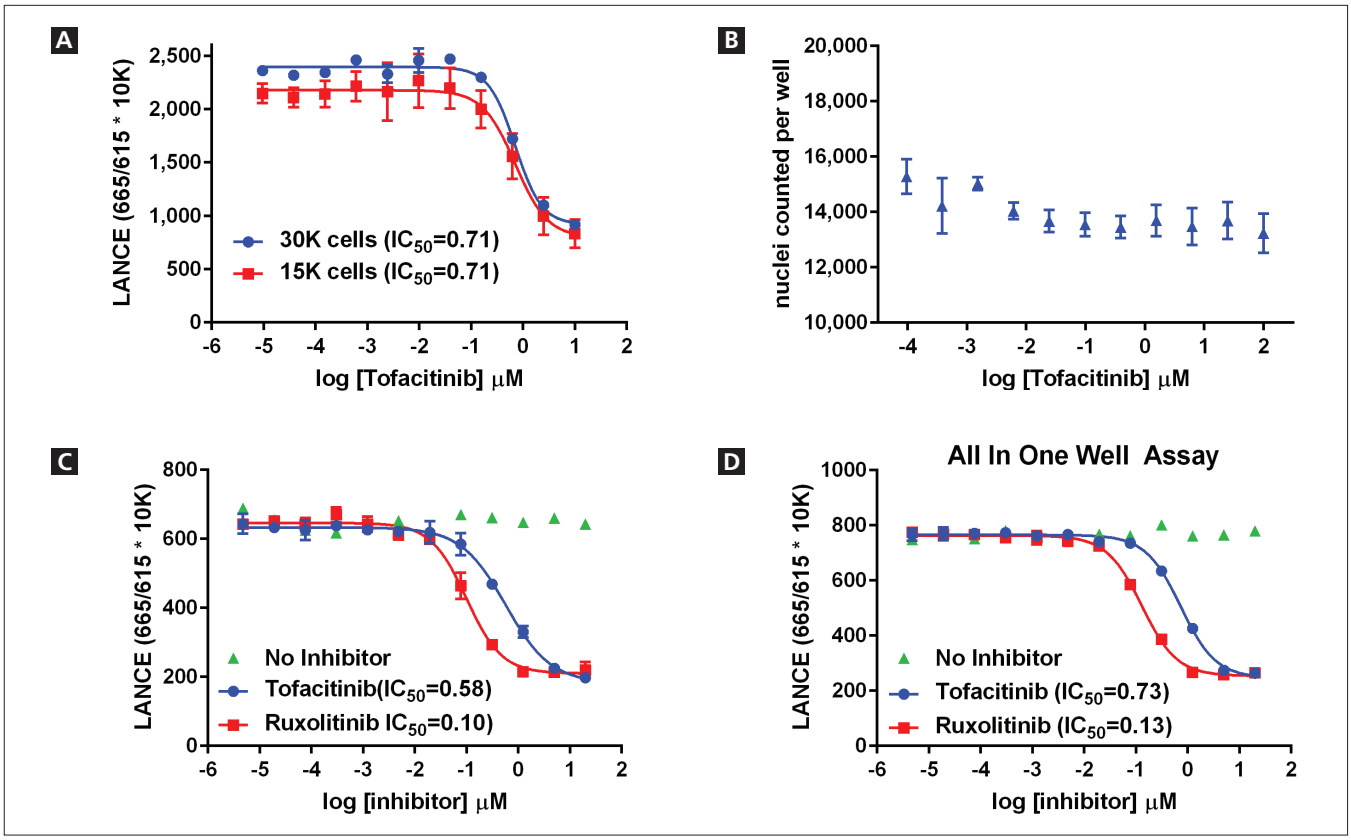

Figure 9. Signaling through the JAK1/2 pathway is necessary for PD-L1 upregulation by IFN-γ. A) A titration of Tofacitinib (a JAK3-specific inhibitor with 20-fold less potency for JAK2) was examined for effect on down-regulating the PD-L1 upregulation by IFN-γ in two concentrations of HCC38 cells. B) To ascertain that cell numbers were not impacted by treatment, cells were labeled with Hoechst, imaged, and counted using the EnSight Count Nuclei function. C) Effects of two different JAK pathway-specific inhibitors, Tofacitinib and Ruxolitinib (a JAK1/2 selective inhibitor) were tested for their ability to inhibit PD-L1 expression in 15,000 HCC38 cells treated with 125 ng/mL of IFN-γ. D) To simplify the assay further, cells were plated and the assay was run in the same wells with 15 µL of assay buffer used for lysis. All data are the average of three wells and IC_{50} values are indicated.

PD-1 Expression in Cellular Lysates

The assays described thus far illustrate the use of LANCE TR-FRET assay technology to detect and quantify PD-L1 expression from cells in culture. Since PD-1 is the primary binding partner for PD-L1 *in vivo*, data in Figure 10 were collected from a titration of lysates made from a HEK293 cells overexpressing PD-1 to illustrate the utility of LANCE for detection of both binding partners in cell culture.

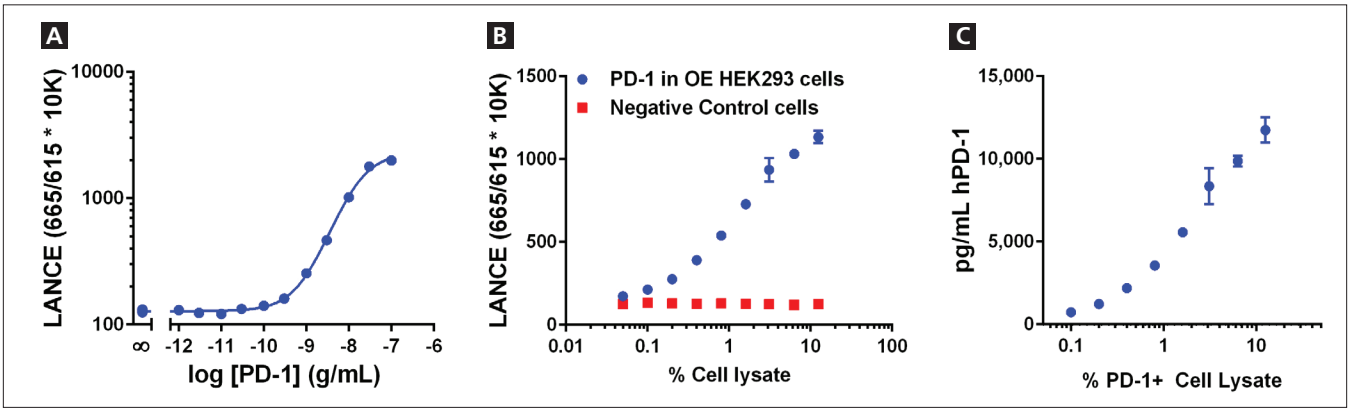

Figure 10. Expression of PD-1 in cell lysates from HEK293 cells overexpressing PD-L1. A) Human PD-1 standard curve generated with the PD-1 LANCE kit was used to measure pg/mL of hPD-1(C) from lysates generated from a HEK293 cell line overexpressing PD-L1 that were diluted in assay buffer (B).

Conclusions

In this application note, we illustrate the power of LANCE *Ultra* assays to detect PD-L1 and PD-1 protein expression levels in human cells quickly and accurately in a no-wash protocol. The data further illustrate that LANCE technology can be used to assess differences in expression between cells from different tumor origins, as shown by comparisons of IFN-γ treatment of two breast cancer cell lines. PD-L1 upregulation via IFN-γ signaling is shown to be controlled through the JAK1/2 pathway as illustrated by down regulation of protein expression in response to treatment with JAK pathway specific inhibitors. We show that LANCE assays provide a fast, powerful, homogeneous platform for screening potential inhibitors of PD-L1 and PD-1 expression in human cells.

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