Rapid, no-wash measurement of immune checkpoint molecules and cytokines in co-cultures of immune cells and cancer cell lines.

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Introduction

Breast cancer tumors can adapt to immune cell infiltration by responding to the increased concentration of interferon gamma (IFN-γ) secreted by subsets of T lymphocytes with the upregulation of the immune checkpoint protein Programmed cell death ligand 1 (PD-L1), allowing the tumors to evade immune targeting and reduce the immune response.[1] Peripheral Blood Mononuclear Cells (PBMCs) are a heterogeneous population of blood cells having round nuclei consisting of monocytes, macrophages, dendritic cells and lymphocytes. In order to examine the effects of interactions between immune and cancer cell populations on the expression and secretion of immune checkpoint proteins and various cytokines, we examined the effects of co-culturing human PBMCs with a triple-negative breast cancer cell line. Specific effects of cell-to-cell contact were examined, looking at effects of secreted factors by including treatment of some wells with PBMC-conditioned media. To induce the differentiation and expansion of T lymphocyte populations in PBMCs, we used Dynabeads®, polystyrene-coated microspheres with antibodies against human CD3 and CD28 proteins covalently coupled to the surface. The binding of the antibodies to CD3 and CD28 proteins on PBMCs mimic the *in vivo* effects of Antigen Presenting Cells (APCs), stimulating cultured T cells to proliferate and further differentiate.[2] Once activated, T cells can upregulate a variety of immune checkpoint molecules and secrete a number of different cytokines in the tumor microenvironment which can have varying effects on specific target cancer cell populations.[3]
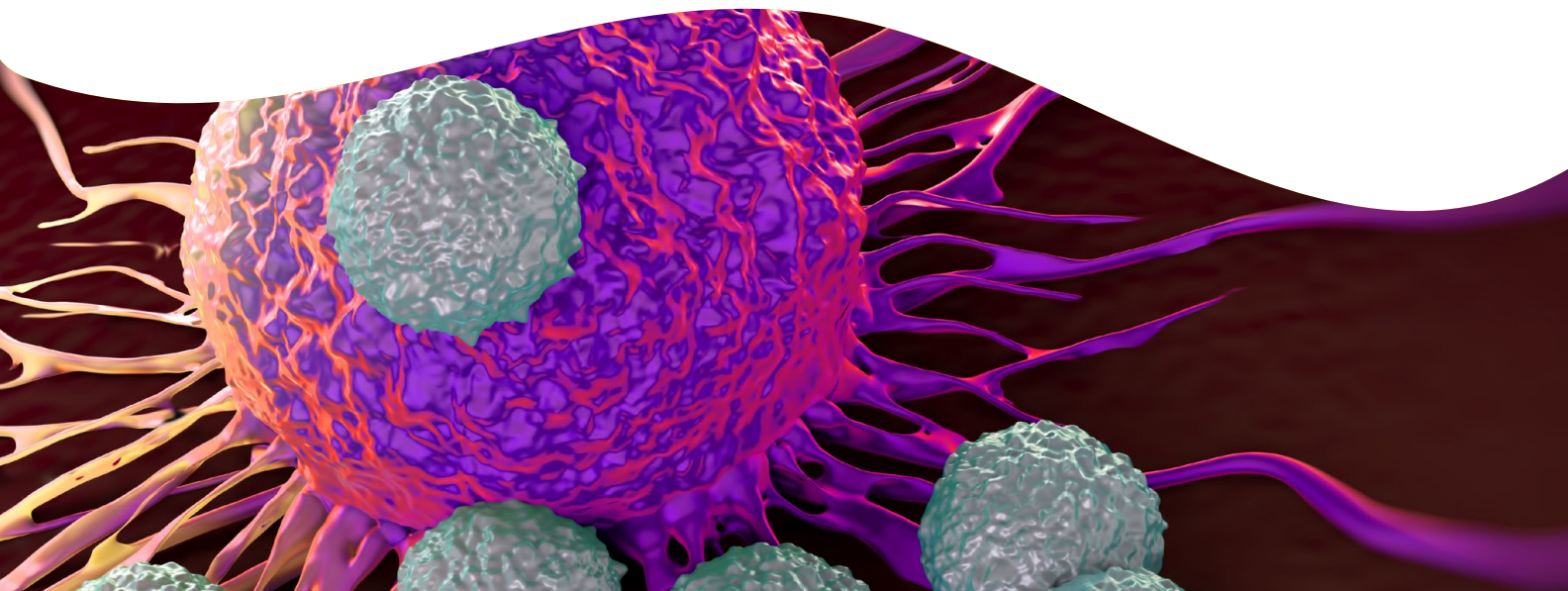
When using traditional wash-based ELISAs, one of the challenges in assessing expression levels of multiple immune checkpoint molecules is the high sample consumption (typically 100 – 200 μL per detection molecule of interest) which can be cumbersome when culturing and treating primary cells such as PBMCs. One way to quickly and easily quantitate multiple biomarkers using a small amount of sample (5 μL per detection molecule of interest) is by using AlphaLISA™ no-wash chemiluminescent technology. Figure 1 illustrates the principle of how AlphaLISA assays are used to detect immune checkpoint markers and secreted cytokines. In Figure, 1A, streptavidin Donor beads bind a biotinylated anti-analyte antibody and another anti-analyte antibody is directly conjugated to the AlphaLISA Acceptor beads. When the analyte is present in the sample, both antibodies bind the analyte and bring the Donor and Acceptor beads into 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 analyte, no signal is generated. An alternate assay set-up, illustrated in Figure 1B and used for detection of most of the cytokines measured here, utilizes digoxigenin (DIG)-labeled antibodies and anti-DIG Donor beads, suggested for use in samples containing high concentrations of endogenous biotin.
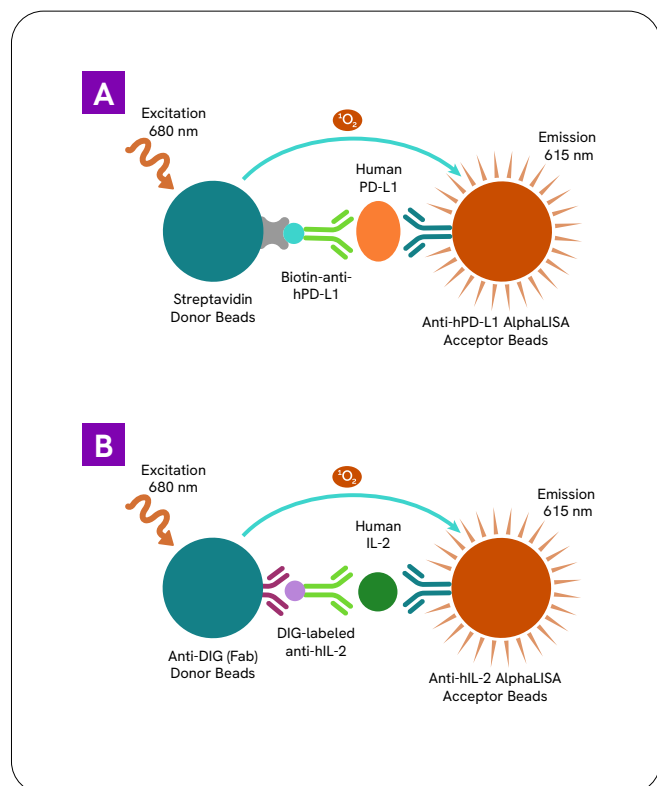

Figure 1. **AlphaLISA assay principle** for detection of (A) immune checkpoint markers and (B) cytokines in biotin-rich media.

Using AlphaLISA we examine the expression levels of immune checkpoint proteins and cytokines in supernatants and cellular lysates from HCC38 (basal breast cancer-derived) cells co-cultured with activated PBMCs, treated with conditioned media collected from activated PBMCs, or directly treated with recombinant IFN-γ. We show how AlphaLISA can be used to detect and quantify multiple immune checkpoint molecules and cytokines in a cell culture model of human breast cancer.

Materials and methods

Cell culture and treatment

HCC38 (ATCC®, #CRL-2314™) cells were grown in culture media consisting of RPMI-1620 (ATCC, #30-2001) supplemented with 10% FBS (ThermoFisher, #26140079) and seeded into 96-well ViewPlates™ (Revvity, #6005182) for experiments. A single vial of normal human PBMCs (ATCC®, #PCS-800-011™) stored in liquid nitrogen was rapidly thawed, rinsed in ice-cold Hanks Buffered Salt Solution (HBSS, ThermoFisher, #14025-134), 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.

For co-culture experiments, the workflow is illustrated in Figure 2. Briefly, HCC38 cells were seeded at 25,000 (25K) cells (in 50 μL) per well on day zero. The same day, PBMCs were prepared at a concentration of 500K cells per mL (equivalent to 25K/ 50 μL) and grown for two days in T-25 flasks (Corning, #353108). Half the flasks were activated by treatment with Dynabead® Human T-Activator CD3/CD28 (ThermoFisher, #11131D) at a standard ratio of one bead to one cell.

After two days, PBMCs were removed from flasks and either added directly to the culture plate containing HCC38, or spun down to collect supernatant for conditioned media (CM). PBMCs or CM were added at 50 μL/well to the plate containing adherent cells. In some conditions, cultures were treated with 100 ng/mL recombinant human IFN-γ (BioLegend, #570206). After two days of co-culture (or treatment with CM or IFN-γ), culture plates were spun down briefly (~4 minutes at ~500 RPM), 62 μL of supernatants were collected (some media was left in order to leave PBMCs in the wells), and cells were lysed by adding 4X AlphaLISA Lysis buffer (Revvity, #AL001C) and shaking (at 600-700 RPM on a DELFIA™ PlateShaker) for 20 minutes. Supernatant and lysate samples (>55 μL lysate per well) were transferred directly to 96-well polypropylene StorPlates (Revvity, #6008290) and kept up to two weeks at -20 °C before AlphaLISA assays were run.
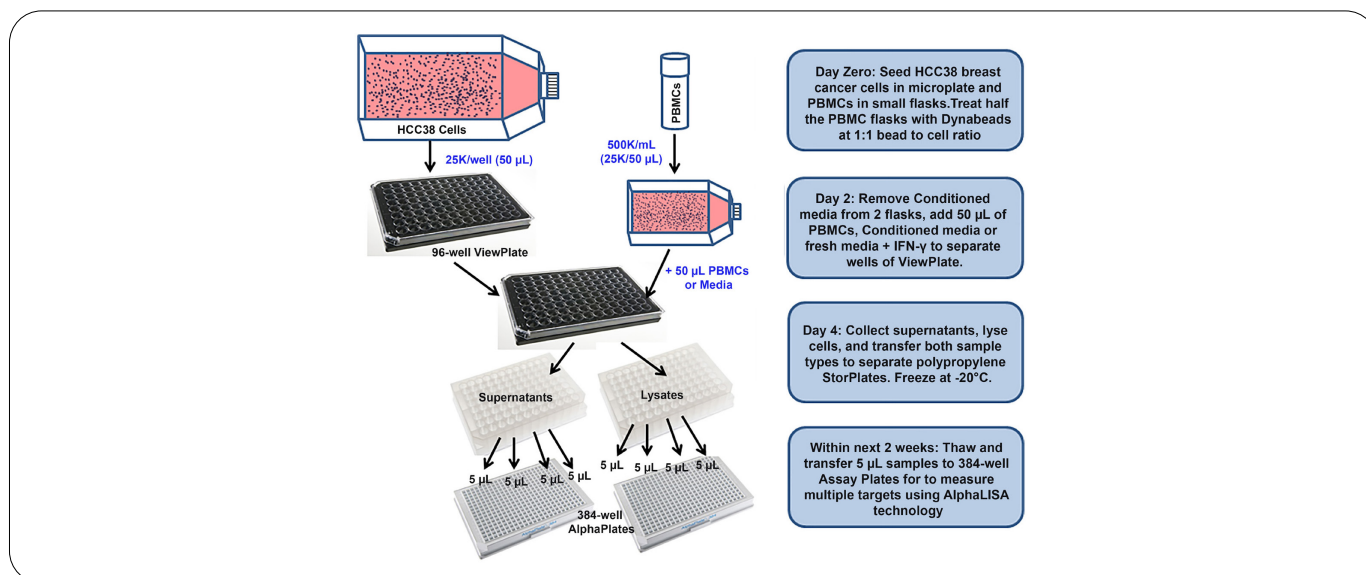
Figure 2. Co-culture assay workflow.

AlphaLISA assays

All AlphaLISA assays were run according to their respective assay manuals. Supernatant and lysate sample plates were thawed and divided into separate AlphaPlate™-384 microplates (Revvity, #6005350) and multiple AlphaLISA kits were run in parallel using 5 µL samples from the same wells of the original culture plate to measure expression levels of various cytokines and immune checkpoint markers. Data collected using AlphaLISA detection kits from Revvity for PD-L1 (#AL355C), IFN-γ (#AL327C), IL-17A (#AL346C), IL-6 (#AL3025C), IL-2 (#AL333C), TNFα (#AL325C), and IL-1ß (#AL220C) are shown. Titrations of recombinant protein standards provided in each kit were run alongside samples being tested in the same sample matrix (culture media or a mixture of media and lysis buffer) and raw data produced were interpolated to the standard curves to determine concentrations (in pg/mL) of each protein measured. Figure 3 shows an example standard curve that was used for quantifying levels of human PD-L1 protein. AlphaLISA is a no-wash, mix-and-read assay technology and most assays were completed in less than three hours. All AlphaLISA assays were measured on the EnVision™ 2105 multimode plate reader using standard Alpha settings.

Data analysis

Standard curves were plotted in GraphPad Prism® using nonlinear regression using the four-parameter logistic equation (sigmoidal dose-response curve with variable slope) with $1/Y^2$ weighting (Figure 3). Protein concentrations were determined by interpolating the counts measured onto the standard curve and all data shown are the average of at least three individual wells. Interpolated analyte concentrations represent the amount of protein in 5 µL of sample. 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.
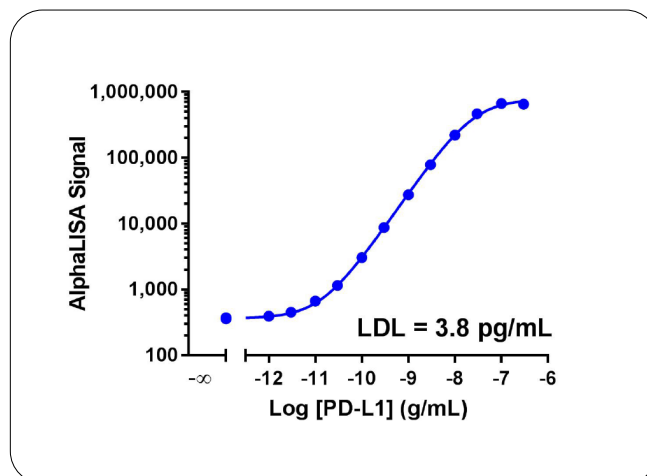

Figure 3. Standard curve for AlphaLISA assay for PD-L1.

ATPlite 1step assays

Cellular proliferation and viability were also assessed in separate wells by examining ATP content in cultures using ATPlite™ 1step (Revvity, #6016731) following the standard protocol. Resulting luminescence was measured with the EnSight™ multimode plate reader.

Results

Pd-l1 expression in co-cultures

HCC38 cells are an epithelial-like, ER-negative cell line and a representative model of triple-negative breast cancer.[4] Previously, we have shown that PD-L1 expression can be induced in cultured HCC38 cells in a concentration-dependent manner with exogenous IFN-γ treatment and measured in cell lysates using AlphaLISA.[5] IFN-γ is one of many cytokines secreted by invading immune cells that can interact with and influence cancer cell populations. In order to further probe the effects of secreted cytokines and differentiate those from the effects of direct contact with immune cells, we developed a co-culture model for investigating the modulatory effects of activated PBMCs on the HCC38 breast cancer cell line. With this culture system, we first examined the effects of activating PBMCs with Dynabeads® on the expression of PD-L1 and compared it to treatment of HCC38 cells with a high concentration of IFN-γ (100 ng/mL).

As previously reported, exogenous addition of IFN-γ alone significantly enhances PD-L1 expression in HCC38 cell cultures (Figure 4, green bar). In addition, we see approximately equal levels of enhancement as a result of treatment with conditioned media (CM) from PBMCs that were stimulated with Dynabeads® for two days. However, direct co-culture with stimulated PBMCs showed greater than 40 times more PD-L1 expression, indicating that the direct addition of activated PBMCs potentiates PD-L1 expression. Much of this difference may be due to the expression of PD-L1 observed in the PBMC population as seen in stimulated PBMCs alone, but the effect is not additive.

Treatment-specific modulation of cytokine secretion

When measuring cytokine concentrations secreted by either HCC38 cells or PBMCs in our culture models, there are a variety of different treatment-modulatory effects observed.
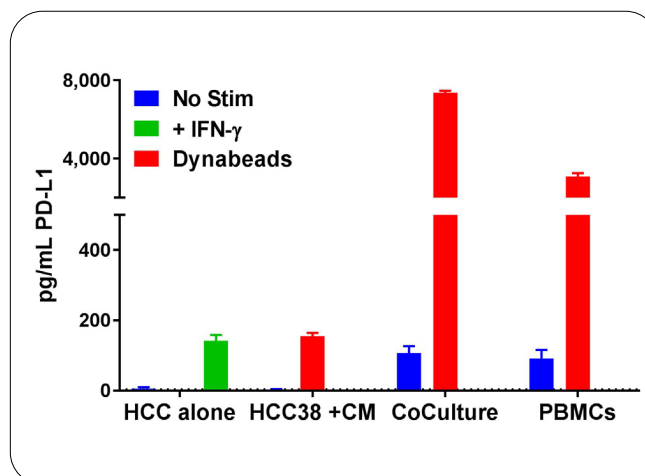

Figure 4. **PD-L1 immune checkpoint protein in co-cultures in lysates**. PD-L1 expression levels in triple-negative breast cancer cell lines and in co-cultures with Dynabead®-activated PBMCs (dotted line = LDL).

As expected, IFN-γ secretion was significantly enhanced in PBMCs activated with Dynabeads® and in cultures treated with conditioned media from activated PBMCs, but that upregulation is surprisingly reduced in co-cultures of PBMCs with HCC38 cells (Figure 5A). Interleukin 17A (IL-17A) levels were elevated only in supernatants from cultures containing HCC38 (Figure 5B) cells that were either treated with IFN-γ or CM from activated PBMCs. Since IL-17A is reported to be a pro-inflammatory cytokine produced and secreted primarily by a subset of T helper lymphocytes, the observation that IL-17A is enhanced in HCC38 cultures alone treated with IFN-γ is very surprising, as there should be no immune cells present in this condition. IL-17A secretion is also reported to be a powerful inducer of other inflammatory cytokines such as TNFα.[7]

Both TNFα and IL-1β expression have been linked to IL-17A secretion and it is, therefore, not surprising that we observed increased levels of both cytokines in the same conditions where IL-17A secretion is observed (Figure 6). However, in co-cultures with stimulated PBMCs, the expression of both TNFα (Figure 6A) and IL-1β (Figure 6B) was enhanced to a greater extent compared to that observed in IL-17A.

As opposed to the expression patterns observed for the cytokines shown so far, IL-6 (Figure 7A) and IL-2 (Figure 7B) were present in the greatest amounts in the direct co-culture conditions stimulated with Dynabeads®. For IL-6, there was enhancement in expression in all stimulation conditions, but the highest levels of IL-6, by far, were present in co-culture supernatants. For IL-2, the *only* detectable levels of protein were present in co-cultures.

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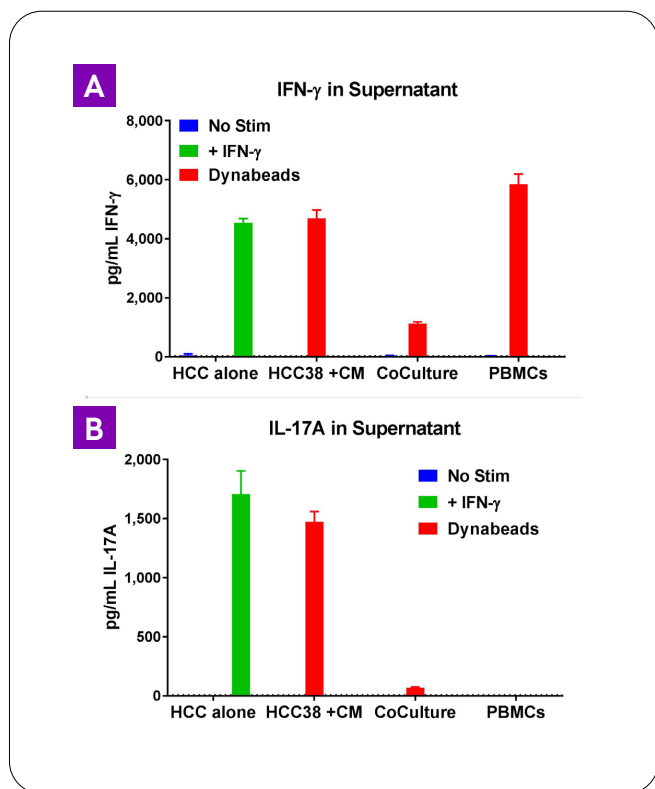Figure 5. IFN-γ (A) and IL-17A (B) levels in culture supernatants show enhancement in different culture conditions (dotted line=LDL).
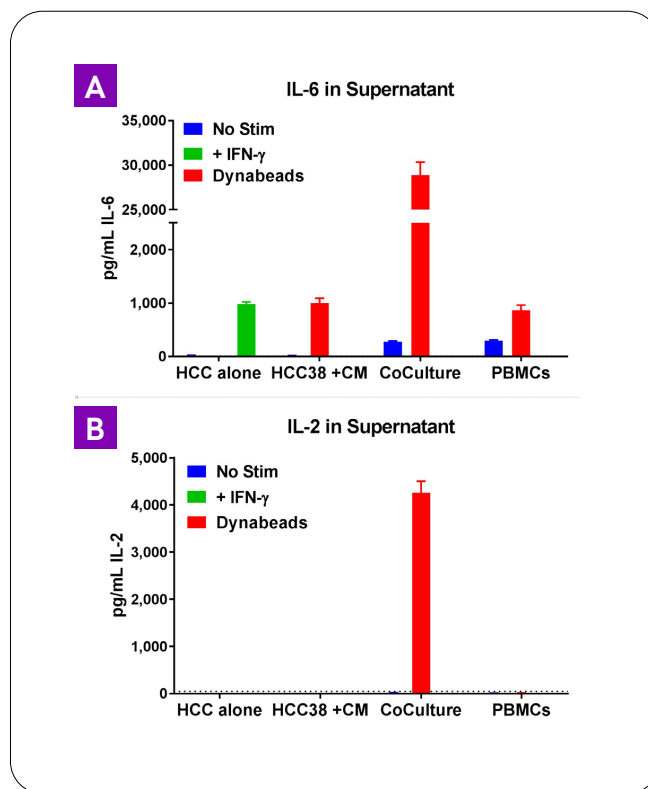

Figure 7. IL-6 (A) and IL-2 (B) in supernatants is most enhanced in co-cultures with activated PBMCs (dotted line=LDL).
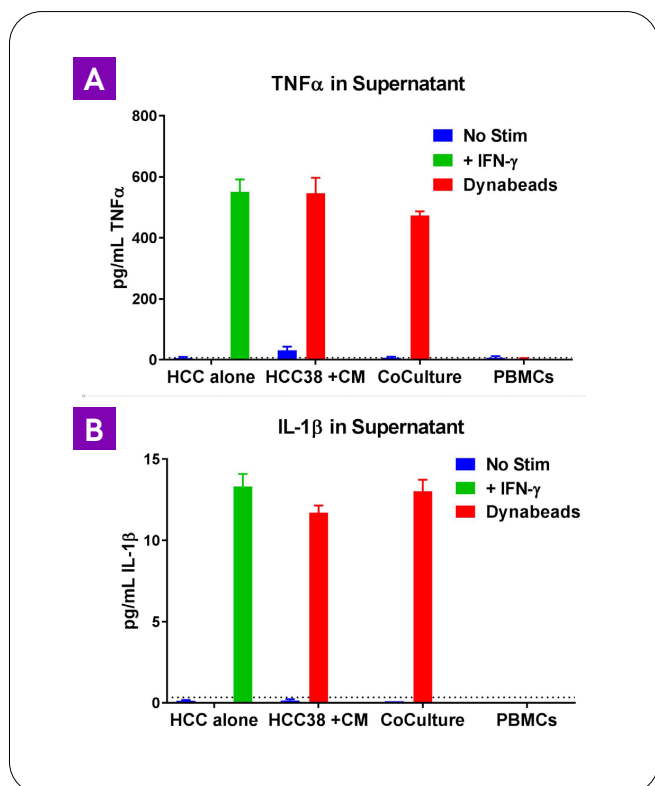

Figure 6. TNFα (A) and IL-1β (B) levels in culture supernatants measured with AlphaLISA (dotted line=LDL).

Effects of treatment condition on cell viability and proliferation

Treatment effects on cellular proliferation and viability were assessed using ATPlite 1step assays and the data are presented in Figure 8. The highest concentrations of ATP were observed in the treatment conditions with HCC38 cells alone. Treating HCC38 cells with CM from Dynabead®-stimulated PBMCs resulted in a reduction in ATP, suggesting that secreted factors from PBMCs were affecting cancer cell proliferation or promoting cell death. Additionally, in PBMCs alone, Dynabead® stimulation resulted in a large enhancement in cell numbers as observed by increased ATP detected and by visual inspection of cultured cells (data not shown). As a control, HCC38 cells alone were cultured and treated for three days with Dynabeads® and no treatment effects were observed with ATPlite 1step or in cell numbers determined by cellular imaging and automated cell counting (data not shown here).[8] The most surprising result observed was the decrease in ATP observed in co-cultures with unstimulated PBMCs. These cultures had the same number of cancer cells to start, so it appears that treatment with unstimulated PBMCs induces cell death in the cancer cell population, more than that observed in Dynabead®-stimulated co-cultures in our model system.
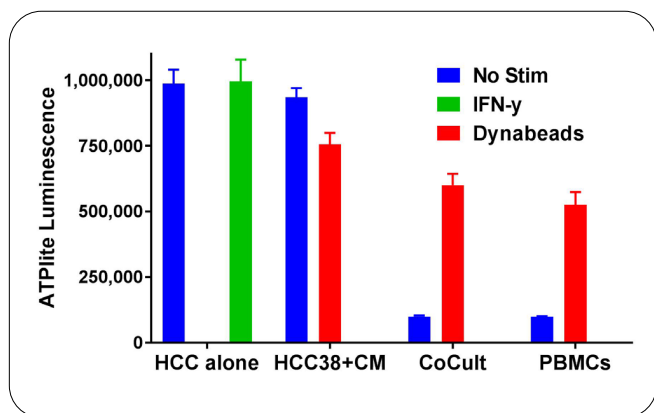
Figure 8. ATPlite assessment of viability and proliferation of HCC38 cells cultured with PBMCs or conditioned media (CM).

Conclusion

We show here that AlphaLISA assays can be used to rapidly measure multiple biomarkers in both cell culture supernatant and lysates from the same wells of a culture dish to examine complex protein expression profiles from cancer and immune cell culture models. Co-culturing activated immune cells and cancer cell lines stimulates differential expression of some immune checkpoint and inflammatory biomarkers compared to culturing cells alone with conditioned media. Many of these inflammatory cytokines interact within the complex tumor microenvironment *in vivo* to effect the expression of different checkpoint proteins that can either promote or inhibit tumor progression. These sorts of complex interactions can be probed further using co-culture models and AlphaLISA assay technology.

References

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