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High Throughput Flow Screening Assays for Immune Checkpoint Inhibitors: Example Assays for PD-L1/2 Functional Blocking Antibodies

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Abstract

The success of immunotherapy approaches has driven emerging interest in the development of antibodies that can target immune checkpoint proteins. However, the complexity of immunology models with the many interacting cells and signaling components necessitates analytical screening tools that can perform not only measurements of cells but also secreted proteins in solution. IntelliCyt's iQue Screener is a high-content, high throughput suspension screening platform that can simultaneously measure cell function endpoints and secreted cytokine levels. Here we demonstrate the flexibility of the iQue Screener for running high throughput flow assays for checkpoint inhibitors by profiling the functional activity of antibodies specific for PD-L1 and PD-L2 both at the level of cytokine secretion and cell-to-cell interaction. The glioblastoma cell line LN229 was utilized in a co-culture model with PBMCs. LN229 cells express PD-L1, which suppresses the activation of T-cells in the PBMC co-culture. T-cell activation was quantified by quantity of IFNg and IL-2 in solution, and results show a 50% reduction of IL-2 and 80% reduction of IFNg. Expression of PD-L1 on LN229 cells was also quantified, and was inversely correlated with the quantity of secreted cytokines. The introduction of blocking antibodies against PD-L1 and PD-L2 (1.25 µg/mL) partially restored the levels of both cytokines, indicating that the PD-L1/2 antibodies effectively blocked the PD-1 signaling pathway and alleviated the suppression of T-cell activation. These results highlight the robustness and flexibility of the iQue Screener for performing multiplexed screening assays for checkpoint inhibitors in complex immunological models.

Introduction

Multiple clinical trials have demonstrated that immunotherapy with antibodies targeting immune checkpoint molecules is an effective method for treating cancer. By modulating co-inhibitory pathways, suppression of T cell activation by tumor cells can be overcome, leading to increased anti-cancer activity by the immune system. An example of this approach involves the interaction between PD-1 on T cells and PD-L1 or PD-L2 on tumor cells, which leads to suppression of T cell anti-cancer activity. Blockade of these interactions restores T cell activation, leading to effective tumor cell killing.

While immunotherapy approaches have seen tremendous success in the clinic, much work remains to be done to develop improved treatments for cancer. In recent years, cell based screening has driven the success of drug discovery programs. Because of the multifactorial nature of immune system responses to cancer, an ideal high throughput discovery platform for immunotherapy approaches requires the following capabilities:

- The ability to perform rapid, plate based experiments on cells in suspension, such as peripheral blood mononuclear cells (PBMC), isolated T and B cells, and other cells of the immune system.
- The simultaneous measurement of cells, cell sub-populations and secreted proteins in the same assay wells provides high content information that can lead to a more complete profile of a treatment's activity.
- Multiplexed detection of secreted proteins to correlate communication via cytokines with cellular activity.
- Scalability to high throughput to enable design of large scale experiments aimed at evaluating tens of thousands of samples in a single experimental campaign.

In this study we describe the use of the iQue Screener and MultiCyt QBeads from IntelliCyt to develop a high throughput screening assay to test the functional activity of antibodies that block the PD-1/PD-L1/2 immune checkpoint axis.

PBMC and Neuroglioblastoma Cell Coculture Model for Checkpoint Inhibitor Functional Screening Assay

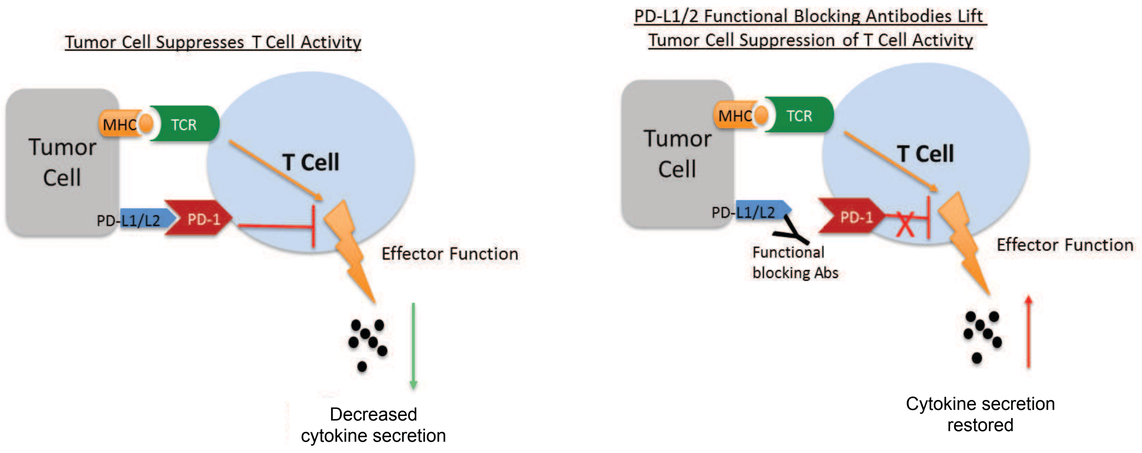

Figure 1A. Assay Model. Tumor cells directly interact with and activate T cells through the T cell receptor (TCR) and induce cytokine secretion such as IL-2 and IFNγ. T cell activation increases the T cell surface expression of PD-1, an immune checkpoint protein which is a receptor for PD-L1/2 proteins. PD-L1/2 expressed on tumor cells binds to PD-1 on T cells and blocks T cell signaling pathways, resulting in decreased cytokine secretion from T cells. Antibodies against PD-L1/2 can block the interaction between PD-L1/2 and PD-1, releasing the inhibitory effects on signaling and increasing cytokine secretion from T cells.

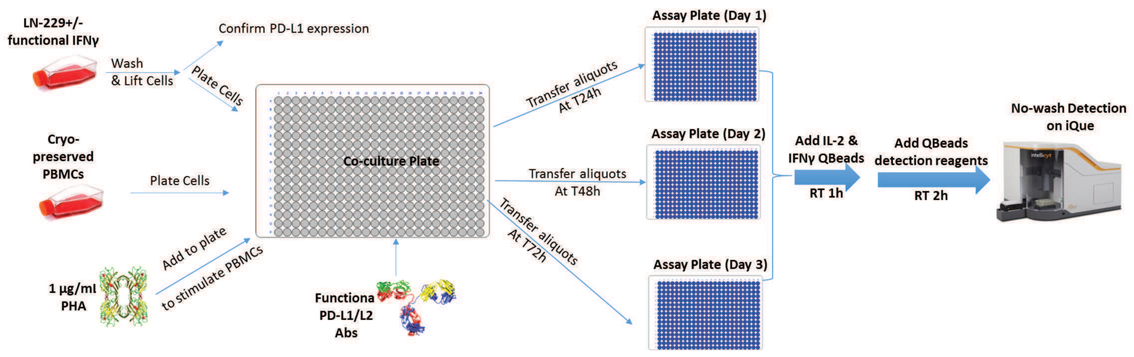

Figure 1B. Screening Assay Workflow. Neuroglioblastoma LN-229 cells with/without pretreatment of IFNγ for 24 hours were lifted/washed and PD-L1/2 expression was evaluated using the iQue Screener. LN-229 cells and PBMCs were co-cultured in 384-well plates (Co-culture Plate) in the presence of phytohemagglutinin (PHA, 1 μg/mL). Anti-PD-L1/2 antibodies were added to the assay plates to block PD-L1/2 binding to PD-1. After 1, 2 or 3 days, an aliquot of the cell suspension from each well was transferred to the assay plates. IL-2 and IFNγ QBeads were added to the assay plates and the plates were incubated for one hour at room temperature. Without washing, the QBeads detection reagent mixture was added, and the plates were incubated for another 2 hours at room temperature. The plates were then directly sampled on the iQue Screener.

IFNγ Induced Expression of PD-L1 on LN-229 Cells

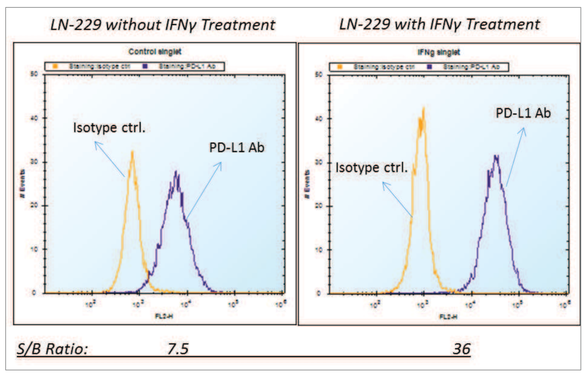

Figure 2. PD-L1 expression was measured on LN-229 cells using the iQue Screener. Cells were incubated with a mouse antibody against the extracellular portion of PD-L1 protein. An isotype control antibody was applied to detect the background staining level. After washing, cells were incubated with a PE-conjugated goat anti-mouse antibody. Cells were lifted by Accutase® (room temperature for 15mins) and then samples were acquired by the iQue Screener. Without pre-treatment of functional IFNγ, LN-229 cells expressed PD-L1 with a signal-to-background (S/B) ratio of 7.5 (A.). Pre-treatment of cells with 1,000 units functional IFNγ for 24 hours increased PD-L1 expression, with a S/B ratio of 36.

Inhibition of PBMC Activation by Coculture with LN-229 Cells and Modulation with Anti-PD-L1/2 Antibodies

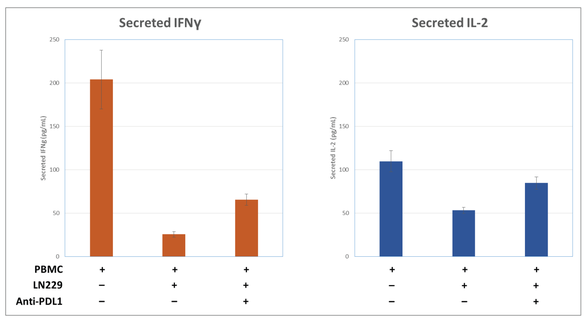

Figure 3. Levels of secreted cytokines IFNγ and IL-2 were quantified with IntelliCyt's MultiCyt QBeads kit. Data shown are from the 48 hour time point of PBMC alone, PBMC cultured with LN-229 cells (pretreated with IFNγ). Cytokine secretion levels of both IFNγ and IL-2 from PBMCs was decreased by introduction of the tumor LN-229 cells into the PBMCs culture. This decrease was partially reversed by treatment with PD-L1 blocking antibody (1.25ug/mL).

Multiplex Assay to Assess Dose Dependent Inhibition of Cytokine Secretion by anti-PDL-1/2 Antibodies

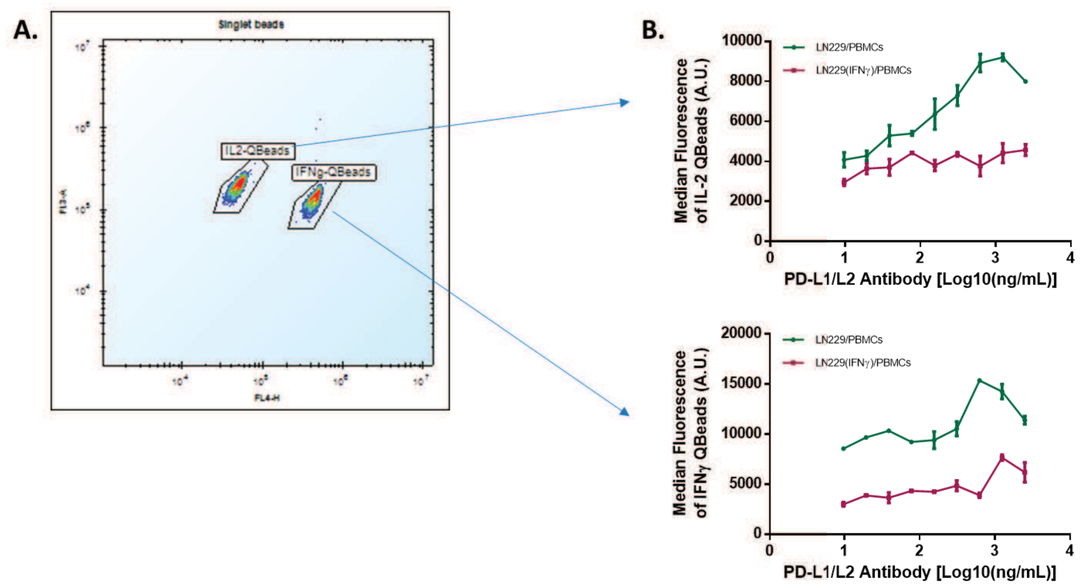

Figure 3. Titration of anti-PD-L1/2 antibodies on cytokine secretion in 48-hour co-culture. Levels of secreted cytokines IL-2 and IFNγ were measured with MultiCyt QBeads following the protocol provided in the kit. Samples were directly acquired on the iQue Screener. **A.** IL-2 and IFNγ capture beads were separated in a 2-D plot based on fluorescence intensity using ForeCyt software included with the iQue Screener. **B.** Dose response of PD-L1/2 antibodies on the secreted IL-2 and IFNγ levels as expressed as median fluorescence detected on the respective capture beads. The effect of the antibodies was tested in cocultures using LN-229 cells that were untreated (green lines) or pretreated with IFNγ to increase PD-L1/2 levels (red lines).

Time Dependent Measurements of Cytokine Secretion Blockade by anti-PDL-1/2 Antibodies

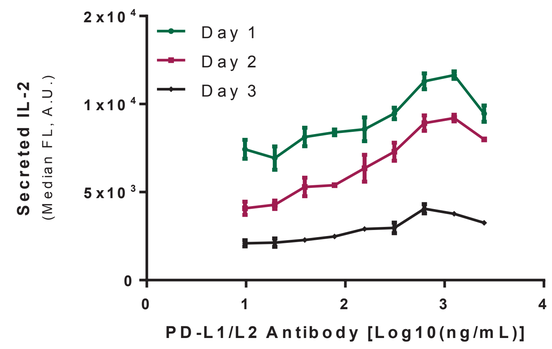

Figure 4. PBMC and LN-229 cells were cocultured in the presence of anti-PD-L1/2 antibodies. At after 1, 2 and 3 days, aliquots from the co-culture plate was transferred to assay plates and levels of secreted IL-2 was measured for each time point using the MultiCyt QBeads kit for IL-2 detection.

Summary

- We demonstrate a multiplexed screening assay to assess the functional activity of antibodies targeting immune checkpoints.
- The iQue Screener, with IntelliCyt's QBeads provides a multiplexed assay solution that can be scaled to high throughput screening, including:
  - Simultaneous detection of multiple secreted cytokines as a readout of PBMC activation at multiple time points.
  - High throughput: Less than 20 minutes to run 384 well plate
  - Low volume assay requirements: The assay was run in 30 μL total sample volume per well
  - Straightforward data analysis of plate level data using ForeCyt analysis software
- Because of the speed of the iQue Screeer and the high content information of this assay, it can easily be incorporated into profiling screens aimed at defining functional activity of checkpoint inhibitor therapies.

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