Analysis of Checkpoint Marker Expression on Immune Cells Cultured with Cancer Cell Lines Using a 10-Color Assay on the BD FACSlytic™ Platform

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Abstract

The immune system plays a critical role in cancer progression. Recently, immune checkpoint-inhibiting drugs have demonstrated significant therapeutic benefit in the setting of melanoma. Much work is being done to explore the power of immune checkpoint inhibitors for treatment of additional cancer types. Immune checkpoint markers include programmed death-1 (PD-1, or CD279) and its ligand PD-L1 (CD274), the proliferation modulator CD82, the costimulatory molecules CD137, CD134, CD28, and its ligand CD86. Accurate measurement of marker expression is critical to patient selection and therapy evaluation. Here we demonstrate an elegant 10-color flow cytometry assay for quantitation of immune checkpoint markers on peripheral blood mononuclear cells (PBMCs) following an in vitro stimulation and coculture with cancer cell lines of multiple lineage. Immune checkpoint surface expression was evaluated using the BD FACSlytic™ platform.

Methods

PMBC Culture Setup and Stimulation

96-well plates were coated with PBS or anti-CD3 (Clone UCHT1) (BD Biosciences Cat. No. 553520) at concentrations of 0.5 μg/mL (Low), 2.0 μg/mL (Med), or 8.0 μg/mL (High). The plates were incubated at 37°C, 5% CO2 for 2 hours and then placed in a 4°C refrigerator overnight. The following day, all wells were rinsed twice with PBS and seeded with PBMC at a concentration of 5 × 10⁶ cells per well suspended in complete DMEM culture media. PBMCs were pooled from three different donors (all normal). After seeding with PBMCs, anti-CD3 coated wells were treated with soluble anti-CD28 or anti-CD86 at a concentration of 2 μg/mL. Wells coated with PBS alone (as a control) were also used. Alternatively, with Phorbol 12-myristate 13-acetate (PMA) at concentrations of 5 ng/mL, 50 ng/mL, or 250 ng/mL or PMA alone. PMA-treated wells were additionally supplemented with 500 ng/mL of ionomycin. Wells were set up in triplicate, and three identical 96-well plates were set up. 96-well plates were incubated at 37°C, 5% CO2 for 24 hours, 24 hours, and 48 hours (data not shown).

Cell Culture and Co-Culture Set-Up

MDA-MB-231 cells (ATCC HTB-26), a human breast adenocarcinoma which was shown based on their high expression of PD-L1 (CD274), were grown to 20% confluence in DMEM (Gibco, W13290010) supplemented with 10% heat-inactivated FBS and 1% Pen/Strep solution. Cells were harvested, washed and counted. MDA-MB-231 cells were mixed with PBMC at a ratio of approximately 3:1 (MDA-PBMC-MB-231). Cultures were stimulated exactly as described above except for PMA, anti-CD28, PMA and ionomycin. Co-cultures were harvested in parallel with the isolated PBMCs described above. Co-cultures were stained and plated to a panel identical to that described in Table 1 (below) with the inclusion of CD632 for cancer cell line identification/elimination of HLA-DR.

Flow Cytometry

After incubation for each of the designated time periods (12, 24, and 48 hours), the cells in each well of the plates were collected and transferred to standard 12 x 75-mm tubes. The cells were washed two times with PBS and recovered via centrifugation (7 minutes at 1000 g). Cells were then stained for 30 minutes with the 10-color antibody panel shown in Table 1 (below). BD Horizon™ Brilliant Stain Buffer (BD Biosciences Cat. No. 556281). After staining, cells were again washed two times with PBS and recovered via centrifugation (7 minutes at 300 g). Samples were analyzed on a BD FACSytec system, and data analysis was performed using the BD FACSlytic™ software platform.

Results

Figure 1. 10-color checkpoint panel graft strategy Isolated PMBC cultures and PMBC cells co-cultured with MDA-MB-231 Cells demonstrated very similar results. A representative set of wells (PMBC cultured alone) is shown here. Cells were cultured for 12, 24, and 48 hours. At the conclusion of each time point, cells were stained with a 10-color flow cytometry panel. CD3+ cells were divided into two main compartments based on CD3 expression. Expression of CD134, CD137, CD247, HLA-DR, CD28, CD152, and CD279 was enumerated on T cells (CD3+) and Non-T cells (CD3-).

Figure 2. Expression of Checkpoint markers following prolonged stimulation PMBCs cultured alone were stimulated with anti-CD3 at concentrations of 0.5 μg/mL (Low), 2.0 μg/mL (Med), or 8.0 μg/mL (High) or with PMA at concentrations of 5 ng/mL (Low), 1.0 μg/mL (Med), or 5 μg/mL (High), or alternatively, PMA at concentrations of 5 ng/mL (Low), 50 ng/mL (Med), or 250 ng/mL (High). PMA-treated wells were supplemented with 500 ng/mL of ionomycin. Stimulated cells were compared against unstimulated controls. Expression levels of all seven checkpoint markers increased in T cells, while the expression levels of these same markers showed increases and decreases in CD3- non-T cells.

Conclusions

We were successfully able to use a 10-color flow cytometry assay on the BD FACSlytic flow cytometer that demonstrates an accurate and reproducible method of determining expression levels of immune checkpoint markers which included CD134, CD137, CD247, HLA-DR, CD28, CD152, and CD279.

The expression of these immune checkpoint markers is an inducible phenomenon that can be controlled through culture conditions and by the choice of stimulatory agent employed.

Expression levels of immune checkpoint markers increased in all states of stimulation (CD3 + CD28, PMA, and PMA + ionomycin) on T cells.

Expression levels of CD134, CD137, and CD247 were increased in non-T cells following stimulation, while expression levels of HLA-DR, CD28, CD152, and CD279 demonstrated moderate decreases in non-T cells following stimulation.

PMA + ionomycin consistently induced the most robust changes in immune checkpoint expression. The effects of PMA and CD3 + CD28 were less dramatic in most cases.

Co-cultures of PMBC and the adenocarcinoma breast cancer cell line MDA-MB-231 which expresses high levels of PD-L1 (CD274) had no effect on the inducibility of checkpoint marker expression as co-cultures showed very similar results to PMBC cultured alone.

References


Table 1: 10-Color Checkpoint Panel

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<th>Phenotype</th>
<th>CD3</th>
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